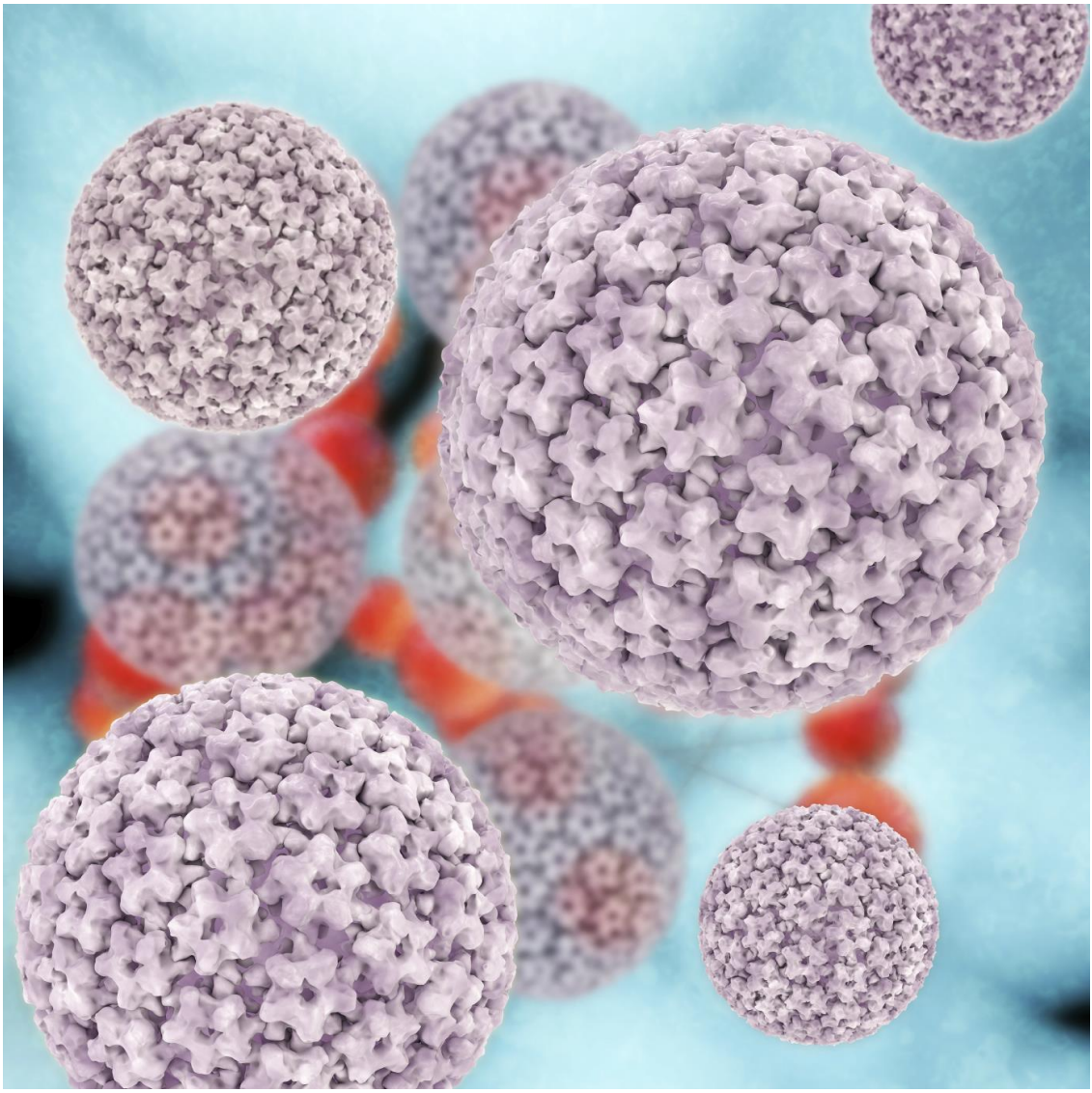

Human papillomavirus in recurrent respiratory papillomatosis, tonsillar and mobile tongue cancer

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Thesis for doctoral degree (Ph.D)
Department of Clinical sciences, Otorhinolaryngology
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Responsible publisher under Swedish law: The Dean of the Medical Faculty.

ISBN: 978-91-7601-509-4
ISSN: 0346-6612-1790
Printed by: Print & Media, Umeå, Sweden 2016
Electronic version of thesis is available at http://umu.diva-portal.org/
Front cover illustration courtesy of Thinkstock. Electronic cover illustration is available at *http://www.thinkstockphotos.com*.
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"You don't develop courage by being happy in your relationships everyday. You develop it by surviving difficult times and challenging adversity."

Epicurus (Greek philosopher of the 4th century BC.)

To my wife and my children, the true meaning of my life.

Table of Contents

Abstract

This thesis focuses on the effects of the human papillomavirus (HPV) in tonsillar cancer, mobile tongue cancer, and recurrent respiratory papillomatosis (RRP). The purpose was to characterize patients with RRP in northern Sweden in order to identify more care-intensive RRP patients and to describe the voice and quality of life aspects that follow RRP. Further aims were to confirm the expected increase of HPV-positive tonsillar cancer cases in northern Sweden, and to study the correlation between HPV, its surrogate marker p16 and HPV receptor syndecan-1 in both tonsillar cancer and mobile tongue cancer.

A total of 27 consecutive patients with RRP were evaluated at 3 months postoperatively using the voice handicap index (VHI) and SF-36 questionnaires to assess the impact on life and voice in a RRP population. The values were compared to normative data. This report was further extended by examining consecutive data from 21 new patients in order to characterize RRP patients in northern Sweden. In order to study HPV DNA in tonsillar (n= 65) and mobile tongue cancer (n=109), HPV DNA was extracted from paraffin-embedded biopsies and detected by polymerase chain reaction using general primers Gp5+/6+ and CpI/IIG. Expression of HPV surrogate marker p16 and the HPV receptor syndecan-1 was analysed by immunohistochemistry.

Patients that underwent more than one RRP surgery per year were younger than those treated less frequently and they had significantly impaired voice quality as compared to normal subjects. Females, patients with frequent surgical treatment sessions, and patients with the high-risk HPV subtypes scored significantly lower in several domains of the quality of life assessment as compared with normal subjects. Forty-eight RRP patients had a median age of 44.5 years; 71% were men and 29% females, preferentially infected with HPV6. Patients with high surgical treatment frequency/year showed more widespread RRP in the larynx compared to the patients treated less frequently.

A total of 214 tonsillar cancer cases were identified. The vast majority were men. They had a median age of 58 years at diagnosis and expressed HPV as well as p16. The incidence of tonsillar cancer revealed a 2,7-fold increase in men between the years 1990 and 2013. The study demonstrates a strong association between p16 and HPV infection in tonsillar malignancies. These findings are in contrast to the mobile tongue cancer cases, where no evidence of HPV DNA could be detected although one-third showed p16 staining. This demonstrated a poor correlation between HPV and p16 in mobile tongue cancer. There was no difference in the expression of the primary HPV receptor, syndecan-1, between tonsillar and mobile tongue cancer.

In conclusion, the frequency of RRP operations, age at onset, gender and subtype of the HPV may be used as factors to predict voice disability. RRP patients with high surgical treatment frequency were significantly younger and had a more widespread laryngeal disease compared to the low-frequency treated group. This study confirms the existence of a clinical RRP group, not primarily related to HPV subtype, but to a more care-intensive RRP population. Our findings identify a 2,7-fold increase in the incidence of tonsillar cancer, HPV and p16 in men between 1990-2013. We can use p16 to detect HPV in tonsillar cancer but not in tongue cancer.

The introduction of vaccination against HPV may have a role in the prevention of specific HPV-subtype positive head and neck malignancies and recurrent respiratory papillomatosis since the current vaccine protects against HPV6, 11, 16, 18, 31, 33, 45, 52 and 58. Males will definitely benefit indirectly from vaccination of females, though males will still remain at risk of cancers associated with HPV. This highlights the need for sex-neutral vaccination strategy. Our intention is that this thesis will provide scientific data to support a gender-neutral vaccination and to develop simple tools to detect HPV in tonsillar cancer.

Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
AoRRP	Adult-onset recurrent respiratory papillomatosis
BSCC	Base of tongue squamous-cell carcinomas
CDK	Cyclin dependent kinases
CMV	Cytomegalovirus
CT	Computer tomography
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EGFR	Epidermal growth factor receptor
ENT	Ear, nose, throat
FDA	Food and Drug administration
FFPE	Formalin-fixed paraffin embedded
FNA	Fine-needle aspiration
GERD	Gastro-esophageal reflux disease
HF	High-frequency group (treated RRP ≥1/year)
HFJV	High-frequency jet ventilation
HIV	Human Immunodeficiency Virus
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
HSPGs	Heparan sulfate proteoglycans
HSV	Herpes simplex virus
ICD	International classification of diseases
IVD	In vitro diagnostic
JoRRP	Juvenile-onset recurrent respiratory papillomatosis
LF	Low-frequency group (treated RRP <1/year)
MCS	Mental component summary score
MGP-PCR	Modified general primer polymerase chain reaction
MHC	Major histocompatibility complex
MICA	MHC class I chain-related proteins A
MICB	MHC class I chain-related proteins B
NASBA	Nucleic acid sequence-based amplification
NBI	Narrow band imaging
NK	Natural killer cell
NKG2D	Natural killer group 2 member D
ORFs	Open reading frames
OSCC	Oropharyngeal squamous cell carcinoma
PCR	Polymerase chain reaction
PCS	Physical component summary score
pRB	Retinoblastoma protein
PRO	Patient-reported outcome

ROS	Reactive oxygen species
RRP	Recurrent respiratory papillomatosis
RT	Radiotherapy
SCC	Squamous cell carcinoma
SD	Standard deviation
SEER	Surveillance, Epidemiology, and End Results Program
SF-36	Short Form (36) Health Survey
SPSS	Statistical Pack for Social Sciences
TORS	Transoral robotic surgery
TSCC	Tongue squamous cell carcinoma
URR	Upstream regulatory region
VLPs	Virus-like particles
VHI	Voice handicap index

Original papers

I. Loizou C, Laurell G, Lindquist D, Olofsson K. Voice and quality of life in patients with recurrent respiratory papillomatosis in a northern Sweden cohort. Acta Otolaryngol. 2014 Apr;134(4):401-6.

II. Loizou C, Laurell G, Lindquist D, Öfverman C, Stefansson K, Nylander K, Olofsson K. Incidence of tonsillar cancer in northern Sweden - impact of human papillomavirus. Oncol Lett. 2015 Oct;10(6):3565-72.

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Sammanfattning på svenska

Syftet med avhandlingen är att beskriva effekterna av humant papillomvirus (HPV) vid cancer i halsmandlarna, cancer i tungan och vid luftvägspapillom.

Totalt 27 patienter med luftvägspapillom (RRP) under åren 2004-2012 utvärderades 3 månader efter operationen med röst handikapp index (VHI) och livskvalitetformuläret SF-36. Resultaten jämfördes med normal data. Studiematerialet utökades med 21 patienter till totalt 48 RRP patienter i syfte att karakterisera patientgruppen i norra Sverige. För att studera HPV-DNA i tonsillcancer (n = 65) och i cancer i mobil del av tungan (n = 109) extraherades HPV-DNA från paraffininbäddade provbitar som sedan analyserades med PCR teknik och GP5 + / 6 + och CPI/IIG primer. Uttryck av surrogatmarkör p16 och HPV-receptorn syndekan -1 analyserades med immunhistokemi.

RRP patienter hade en medianålder på 44,5 år; 71% var män och 29% kvinnor, företrädesvis infekterade med HPV6. Patienter som opererades mer än en gång per år var yngre än de som behandlats mindre ofta och hade en statistiskt sämre röstkvalitet än friska kontroller. Kvinnor, patienter med täta kirurgiska behandlingsintervall och högrisk-HPV hade signifikant sämre livskvalitet jämfört med friska kontroller. Patienter med hög kirurgisk behandlingsfrekvens per år var signifikant yngre och hade mer utbredd RRP sjukdom i luftstrupen, jämfört med gruppen med låg behandlingsfrekvens.

Sammanlagt, 214 fall av halsmandelscancer identifierades i norra Sverige under åren 1990-2013; majoriteten var män, med en medianålder på 58 år och positiva för både HPV och p16. Andelen HPV-positiva halsmandelscancer fall ökade med 2,7 gånger bland männen på 23 år. Vi fann ett starkt samband mellan uttryck av p16 och HPV infektion i halsmandelscancer men inte i HPV-negativ, delvis p16-positiv (33%) mobil tungcancer. Det fanns ingen skillnad i uttrycket av den primära HPV-receptorn, syndekan -1, jämförande tung-, och halsmandelscancer.

Antalet RRP operationer, ålder vid insjuknandet, kön och genetisk variant av HPV kan användas som indikatorer för att förutsäga grad av röststörning. RRP patienter med hög kirurgisk behandlingsfrekvens var signifikant yngre och hade en mer utbredd luftvägssjukdom jämfört med RRP patienter som behandlas mindre ofta. Vi har identifierat en undergrupp av RRP patienter som inte primärt karakteriseras efter HPV virusets genetik utan av ett mer vårdintensivt förlopp. Den aktuella avhandlingen har identifierat en 2,7-faldig ökning av antalet halsmandelscancer hos män och ett starkt samband mellan p16 och HPV infektion i halsmandlar men inte i HPV-negativ tungcancer som inte korrelerar till p16 uttryck. Vi kan använda p16 för att påvisa HPV i tonsillcancer men inte i cancer i mobil tunga.

Idag ingår HPV vaccination i det allmänna vaccinationsprogrammet för flickor. Vi förväntar oss en tydlig profylaktisk effekt avseende insjuknande i HPV-relaterad huvud- och hals cancer samt luftvägspapillom eftersom vaccinet skyddar mot HPV bl.a. 6, 11, 16 och 18. Män kommer definitivt att gynnas indirekt genom vaccination av kvinnor men kommer att ha fortsatt högre risk än kvinnor att insjukna i HPV relaterad cancer vilket understryker behovet av könsneutral vaccination. Vår avsikt med avhandlingen är att ge vetenskapligt stöd för könsneutralt vaccination och enkla metoder att påvisa halsmandelscancer.

1. Introduction

1.1 Human papillomavirus

In the 1970s, Harald zur Hausen detected the human papillomavirus (HPV) in warts and cervical cancer. He published the hypothesis that HPV could play an important role in the pathogenesis of cervical cancer. He isolated and cloned different strains of HPV, and concluded that infection with HPV16 and 18 constituted an increased risk of developing cancer. (1) For these findings, as well as the fact that subsequent to these descriptions, HPV vaccines were developed in order to prevent cervical cancer, Dr. Hausen received the Nobel Prize in Physiology and Medicine in 2008. Currently, HPV is the most common sexually transmitted disease, (2) with close to 200 genotypes identified, and more than 80 genomes which have been completely sequenced. (3) HPV infection is common and most infected individuals are able to successfully eliminate evidence of the virus without presenting any manifestations of clinical disease. The classification of the HPV genotypes has been frequently revised, with current data supporting the idea that 15 genotypes are oncogenic (for example HPV16, 18, 31, 33, 45, 52 and 58), 3 subtypes are considered high-risk, 12 low-risk, and 3 are referred as undetermined risk genotypes. (4) HPV infects the squamous epithelium of the skin (subtypes 1, 2, and 4), the genital mucosa (condylomata acuminata, subtypes 6 and 11), and the upper airways (subtypes 6 and 11).

1.1.1 Epidemiology

The reported prevalence of HPV infection among women around the world ranges from 2% to 44%, probably due to differences in the age range of the population studies and the methods used for the detection of the virus. (5) HPV infection in men has not been studied as extensively as in women, but the prevalence varies between 16,5% (6) and 32,7%. (7)

1.1.2 Taxonomy

Human papillomavirus belongs to the family Papillomaviridae. The HPV viruses are small, non-enveloped, double-stranded, circular DNA viruses that have a particular tropism for the epithelium. (3) They are highly species-specific. The taxonomic classification is based on sequence variations in the L1 open reading frame and the taxonomic levels related to ’families’, ’genera’, ’species’, ’types’, subtypes’, and ’variants’. (8) Despite their small size, their molecular biology is complex. The different proteins of the late (L) and early (E) regions have specific functions.

1.1.3 Genomic organization

The human papillomavirus genome contains a double-stranded circular DNA with approximately 8000 base pairs. (8) These are functionally organized into two

regions: Upstream Regulatory Region (URR) and Open Reading Frames (ORFs) (Figure 1). The URR is a non-coding region, which regulates the expression of viral gene. The ORFs can be divided into two coding regions; the late (L, codes for the L1-L2 capsid proteins which comprise the outer coat of the virus) and the early (E, codes for the E1-E2 and E4-E7 proteins which are necessary for the replication, cellular transformation, and the control of viral transcription). (9) The E1 and E2 viral proteins play a significant role in DNA replication. The E1 protein is the largest and most conserved; it is a 68 kDa protein, which is necessary for DNA replication.

1.1.4 Viral proteins and viral genome integration

The E1 protein binds to specific DNA elements during the viral replication process and assembles into hexameric helicases with the aid of the 50 kDa *E2* viral protein. The resultant complex provides the template for subsequent DNA syntheses through an initiation of origin DNA unwinding. (9, 10) Furthermore, E1 protein binds to DNA polymerase during basal replication and interacts with cyclins A and E, (11) while E2 protein acts as a transcription factor whose functions can be disrupted by mutation or integration of the viral genome. As a consequence, the expression of E6 and E7 genes are increased, enhancing carcinogenesis and malignant progression. (12, 13)

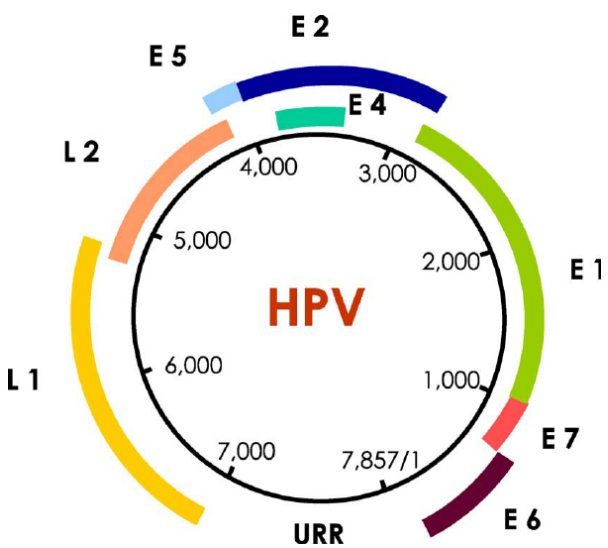

Figure 1. Schematic presentation of the HPV genome. Reproduced by kind permission of Rightslink/Elsevier. (14)

E4 is a 17 kDa protein contributing to genome amplification efficiency and viral synthesis. This is done through the induction of G2/M arrest function and with a particular role in the latest phase of the viral cycle life. Furthermore, it can serve as a biomarker of active virus infection and disease severity, especially in the case of high-risk HPV genotypes. (15, 16)

E5 protein is one of the oncoproteins expressed by the HPV genome, enhancing the activation of the epidermal growth factor receptor (EGFR) after stimulation by EGF in human keratinocytes. (17) E5 can augment the function of E6 and E7 proteins in the transforming activities of HPV viruses, contributing to tumor progression when expressed alone. E5 has a weak transforming activity. (18) Another suggested function is the inhibition of localization of the major histocompatibility complex (MHC) class I and II proteins to the plasma membrane. (19)

E6 protein is expressed early in the viral cycle and, along with E7, is one of the main oncogenic proteins. This acts by stimulating the growth and transformation of the host cell through the inhibition of protein p53's normal tumor suppressing function. The cell proliferation activity is suggested to be the result of the formation of a trimeric complex including E6, p53, and the cellular ubiquitination enzyme E6-AP. The formation of this complex leads to degradation of the tumor suppressor p53 protein. (20) The complex consists of 158 amino acid residues and contains two zinc-finger binding motifs. (21) Another suggested function is the increase of telomerase activity in epithelial cells, a feature observed in normal proliferating tissues and malignancies. (22)

E7 protein is also expressed early in the viral life cycle, as mentioned above. It is a small protein consisting of 98 amino acids, and is thought to have the major transforming effects. More specifically, E7 acts by binding to members of the retinoblastoma (Rb) tumor suppressor protein family, and induces cellular proliferation by release of the E2F transcription factor. (23) Several other functions of E7 have been proposed; the promotion of HPV replication by direct activation of cyclin-dependent kinase 2, (24) activation of hypoxia-inducible factor 1-mediated transcription by inhibiting binding of histone deacetylases, (25) and suppression of cadherin-mediated cell adhesion via the ERK and AP-1 signalling pathways. (26)

The L1 major capsid protein supports the viral capsid, together with L2. It self-assembles into pentameres, and 72 pentameres self-assemble into virus-like particles (VLPs). The ability of VLPs to elicit neutralizing antibodies has been used for the development of prophylactic vaccines that can prevent persistent HPV infection-associated malignancies. L2 is the minor capsid protein, not required for VLP formation and in accordance with L1. It is only expressed in terminally differentiated epithelial cells. Proposed functions of this protein are the interaction

with the viral genome and encapsidation of viral DNA. (27) Furthermore, it facilitates the infection of cells by HPV through an interaction with an unknown cell surface receptor. (28)

Viral genome integration

The virus is easily transmitted, possibly through microscopic tears in the surface of the epithelium of the skin or mucosa. An HPV infection is strictly limited to the basal cells in the mucosa or skin. The virus interacts with the surface of the cell via interaction of the L1 major capsid protein with heparan sulfate proteoglycans (HSPGs) on segments of the basement membrane. There is also accumulating evidence for the role of secondary receptors such as alpha-6-integrin and the L2 minor capsid protein during the binding process. (29, 30) In the majority of human cervical carcinomas, the HPV genome is integrated in the host genome, which frequently leads to disruption of the E2 gene regulating the expression of E6 and E7. (31) There has been discordance between different studies that evaluated the integration of HPV virus in oropharynx/tonsillar cancer, identifying the presence of integrated, (32, 33) episomal, (32-35) or mixed (32) HPV DNA. The results from those studies have not been widely confirmed though; the question whether the virus is integrated or episomal remains unanswered with conventional consensus primer-based PCR.

Replication occurs within the nucleus of the infected cells, and requires DNA mechanisms that are partly controlled by E1 and E2 proteins. (36) The differentiation of the squamous epithelial cells takes place as they move from the basement membrane towards the epithelium of the surface. Replication of the viral DNA in high numbers occurs only in terminally differentiated cells near the surface layers. Similarly, the expression of the L1 and L2 proteins that form the virus particle is also encoded in these highly differentiated cells by the late viral genes. (37, 38)

HPV and cancer development

High-risk human papillomaviruses produce the oncoproteins E6 and E7, which play a major role in malignant transformation through their proliferation-stimulating activity. The E6 protein binds and induces the degradation of the p53 tumor suppressor protein through ubiquitin-mediated proteolysis, leading to substantial loss of p53 activity and uncontrolled cell cycle progression. (39) On the other hand, the E7 oncoprotein binds and inactivates the retinoblastoma protein (pRb), allowing the transcription of E2F-dependent genes. This causes the cell to enter S phase and leads to loss of cell cycle control. The inactivation of pRb results in overexpression of the p16 protein, which is encoded by the CDKN2A tumor suppressor gene. The latter explains why p16 overexpression is associated with high-grade precancerous lesions and carcinomas. This demonstrates the value of immunohistochemical

evaluation of p16 as a surrogate marker in identifying HPV infection in cancer cells. (40, 41)

The correlation between p16 and HPV in malignant tumors appears to be site-specific, and must therefore be charted individually. (42, 43) Furthermore, the oncoproteins E6 and E7 can lead to DNA mutations of the host cell through alterations of DNA repair mechanisms. This can explain how specific HPV genotypes can induce malignancy without any other co-factors. (44) The presence of the oncoproteins E6 and E7 is not enough to explain the HPV-induced carcinogenesis that often requires decades to develop. Additional cellular changes and risk factors previously named are often present during the process of initial hyperplasia of the normal epithelium, which can proceed to dysplasia, carcinoma in situ, and ultimately development of invasive cancer.

1.2 Human papillomavirus and human disease

1.2.1 HPV and benign lesions

Recurrent respiratory papillomatosis (RRP)

The association between RRP and HPV infection was first postulated in the 1920s. (45) It is now well-recognised that persistent HPV infection, mainly HPV6 or HPV11, is the primary cause of RRP. The presence of additional co-factors, some yet unknown, may be required.

HPV in trachea

Tracheal spread of RRP has been proposed in 16% of adult-onset RRP (AoRRP) and in 30% of juvenile-onset RRP (JoRRP). (46) RRP affecting the larynx has a higher tendency of remission than RRP localized in trachea and/or bronchi. (47)

Non-visible HPV in larynx

Although RRP is thought to be rare, the airway is constantly exposed to HPV throughout an individual's life. HPV6 has been detected in healthy laryngeal mucosa in 25%, and the amount of HPV6 DNA in RRP patients is consistently higher in the papilloma lesion than in healthy adjacent mucosa. (48-50)

HPV in oral cavity

HPV is identified in the oral cavity in less than 7% of the general population. This colonization rate is much higher in patients with AoRRP and their long-term sexual partners. (51) The oral HPV prevalence in Sweden (2009-2011) among HPV-unvaccinated young people aged 15–23 years was 9,3%. (52)

HPV in genital warts
Condylomata acuminata is a benign disease, sexually transmitted and caused by HPV6 and 11. Women with genital warts have an increased risk of cervical carcinoma and the lesions are associated with local clinical symptoms such as burning, bleeding and pain. (53)

1.2.2 HPV and malignant lesions

The high-risk subtypes of the HPV virus are more likely to cause malignancies. Some individuals develop a long-lasting HPV infection that causes changes leading to precancerous lesions or cancer.

Cervical cancer
Cervical cancer in a global perspective is the 4th most common form of cancer and the 4th most common cause of female cancer death. (54) An estimated 528 000 cases of cervical cancer occurred in 2012, with an approximate 266 000 patients deaths. This means that cervical cancer constitutes approximately 8% of all cancer-related death in women. (54) HPV16 and 18 account for 70% of all cervical cancer, with some regional variations worldwide. HPV16 has been detected in 24% of women infected with HPV, while 9% are infected with HPV18. (55, 56)

Head and neck and oral cancer
The majority of HPV infections are asymptomatic and are cleared spontaneously. It is uncertain why some HPV infections lead to permanent infections, thus creating conditions for developing carcinoma, and in specific cases requiring lifelong treatment. In Finland, HPV-related oropharyngeal cancer incidence has almost tripled over the last 30 years. (57) The increase in oropharyngeal and oral malignancies is noted worldwide, affecting young non-smokers and non-drinkers. Smoking and drinking have been considered as the main risk factors for head, neck and oral cancers. Besides tonsillar cancer, HPV has been associated with cancer of the base of the tongue (BSCC) in 40% to 75% (58, 59) and laryngeal carcinomas with a lower incidence. (60, 61) Certain molecular profiles of HPV-related head and neck squamous cell carcinomas (HNSCCs) are different from HPV-negative cancers. These can be biologically distinct entities where the HPV-positive HNSCC cancers have a better prognosis. (62) Most HPV-associated HNSCCs are caused by HPV16, and tend to present mostly at an early T stage and advanced nodal stage. (63)

Anogenital cancer
The etiological role of high-risk HPV has also been studied in penile cancer (23%-48%), (64, 65) anus (90%), vagina (40%) and vulva (40%). (66) The full extent of the association between HPV and these cancers in terms of age and onset of diagnosis is not well known. Further studies are needed in order to clarify this issue. The introduction of vaccination against HPV has been shown to have utility in the

prevention of HPV-related anogenital cancers. (66)

Lung cancer
Syrjänen first reported (1979) that HPV could possibly be involved in bronchial squamous cell carcinoma. (67) Several studies have reported a role of HPV in the development of squamous cell carcinoma of the lungs. The mean incidence of HPV in lung cancer is 24,5%. (68) The incidence is heterogeneous and the diversity related to the "home country" is about 17% in Europe and 15% in the U.S., while HPV was present in 35,7% of the Asian lung cancer samples. (68)

1.2.3 Risk factors linked to HPV

Behaviourally-based risk factors linked to HPV include an increasing number of lifetime sexual partners and partner characteristics such as age, ethnicity and smoking, (69) the use of oral contraceptives, (70) alcohol and illicit drug use, (71) and diet. (72) On the other hand, biologically-based risk factors include age <15 years at first sexual intercourse and age at first menarche, (71) immunosuppression, concurrent HIV infection, and occurrence of other sexually transmitted infections. (73, 74)

1.2.4 HPV and the immune response

HPV-infected individuals develop an ineffective HPV-specific T-cell immune response. Recent studies have shown that HPV infections create a Th2-like immune response in which CD4+ T-cells induce expression of immunosuppressive cytokines (e.g. IL-4 and IL-10) that suppress Th1 (cytotoxic) immunity. (75) The RRP lesions have been found to express a parallel decrease in IFN-γ, IL-12 and IL-18. (76) Elevated Th2-like cytokine response has been suggested to correlate with disease severity. (77) This imbalance of Th1/Th2-like cellular response could explain why RRP patients fail to prevent the recurrences of the disease. The expression of major histocompatibility complex (MHC) antigens seems to play a significant role in patients with RRP through altered function of cell-mediated immunity. MHC class I chain-related (MIC) A and B molecules, ligands of the activating NK-cell receptor NKG2D, and stress-induced molecules were shown to be up-regulated and exhibited differential expression among HPV-infected and non-infected cell lines. (78) Dysfunctional natural killer (NK) cells are present in RRP lesions, but are unable to clear the infected HPV keratinocytes. (79) A summary of the immune responses to HPV is shown in Figure 2 below.

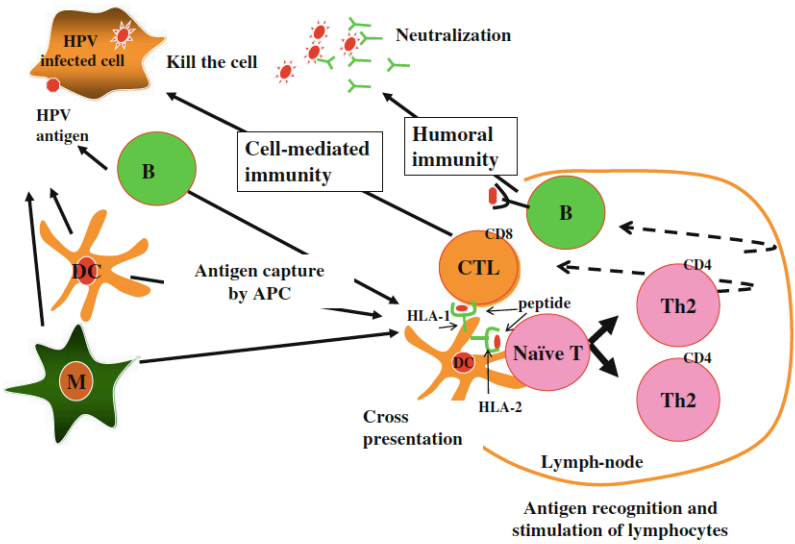

Figure 2. Immune responses to human papillomavirus. (DC) dentritic cell, (CTL) cytotoxic T lymphocyte, (APC) antigen-presenting cell, (M) monocyte, (B) lymphocyte. Reproduced by kind permission of Dr. Sasagawa T. (80)

The NKG2D receptor-ligand system constitutes an important cytotoxic effector mechanism in elimination of infected, foreign, stressed and/or transformed cells. The engagement of NKG2D with a ligand could bypass inhibitory signals from other NK receptors, and lead to destruction of the NK-cell target. (81) The deficiency of the system NKG2D receptor-ligand has been shown to promote the development of spontaneous tumors in mice. (82) The NKG2D ligands serve as antigens that can induce cell proliferation, cytotoxicity, and cytokine production after linking to the appropriate NKG2D receptor. (83) NK-cell triggering by NKG2D ligands induces CD25 expression, NK-cell proliferation, cytokine production, and perforin-mediated cytotoxicity. This results in killing of target cells that express these ligands. (81, 84)

1.3 HPV vaccines

Recently, the introduction of vaccination against HPV-associated anogenital cancers in non-infected subjects has revealed a promising amount of HPV protection. These prophylactic HPV vaccines contain the major capsid protein L1 that self-assembles into immunogenic virus-like particles similar to authentic virions though they are non-infectious. (85) Currently, two HPV prophylactic vaccines have been successfully developed: the bivalent Cervarix® (GlaxoSmithKline, Rixensart, Belgium) and Gardasil® (Merck & Co., Whitehouse Station, NJ). Both are highly efficacious for the prevention of persistent infection with HPV16 and 18 in cervical

lesions. (86) Two-dose schedule is currently recommended by WHO (2014) and applies in Sweden since January 1, 2015 for girls aged 9-13 years. For women 14 years and older (Cervarix® 15 years and older), a three dose-schedule is recommended. The vaccines are given as intramuscular injections. Gardasil 9® is a 9-valent recombinant vaccine against human papillomavirus, and a further refinement of Gardasil®. The major change in Gardasil 9® is the inclusion of boys for the prevention of diseases caused by HPV genotypes 6, 11, 16, 18, 31, 33, 45, 52, and 58. The vaccine has the ability to protect against 90% of cancers of the cervix, vulva, vagina, and anus. It is approved in the United States against these forms of cancer, and against genital warts in females 9-26 years and boys 9-15 years. (87, 88)

A growing number of countries recommend or permit HPV vaccination for males. Cost-benefit analyses have concluded a clear benefit of expanding HPV vaccination programs including males. (89) Prophylactic vaccination against high-risk HPV genotypes has been proven effective in the prevention of genital HPV infection, and consequently genital HPV-related malignancies. Despite this, there is no proven efficacy in the prevention of HPV-related tonsillar cancer or recurrent respiratory papillomatosis. (90, 91)

Despite the effectiveness of these vaccines, several issues still need to be addressed. The main challenge is delivering these vaccines to patients in low- and middle-income countries, owing to the high cost of the vaccines. Furthermore, immunity to several high-risk HPV genotypes is not provided by these vaccines. Additionally, whether or not a booster dose is needed is not known.

Currently, efforts are directed towards strategies that could make therapeutic HPV vaccination possible. These strategies can elicit a cytotoxic $CD8^+$ T-cell response against high-risk HPV E6 and/or HPV E7 oncoproteins in both humans and mice. (92) In mice, transplantable tumors that express these viral antigens have been successfully decreased in size by this treatment strategy, but this approach is not yet applicable in humans. (93, 94)

1.4 HPV DNA detection methods

It is not possible to propagate or isolate HPV in tissue culture, which is a common method for virus detection. HPV can be detected by identification of proteins of HPV genomic sequences in the infected tissue, or indirectly by measuring specific IgG antibodies in the serum against virus-specific antigens from earlier infections. The most commonly used detection method is based on direct hybridization or DNA amplification techniques, polymerase chain reaction (PCR). Additionally, detection of HPV E6/E7 mRNA can be performed by reverse-transcriptase PCR.

Polymerase chain reaction, PCR
PCR is a biochemical technology used to amplify a piece of DNA, generating multiple copies of a particular DNA sequence. It is currently regarded the most sensitive technique allowing tests on samples with only small amount of tissue available.

Quantitative PCR
Q-PCR is also referred as real-time PCR, and is used to quantify the target DNA molecule. Q-PCR uses a DNA binding dye, causing fluorescence. An increase in the DNA product during PCR leads to increased fluorescence intensity, measured at each cycle, allowing DNA concentration to be assessed.

PapilloCheck®
The DNA array PapilloCheck® (Greiner Bio-One GmbH, Frickenhausen, Germany) is certified in the European Union (CE) as an *in vitro* diagnostic method (IVD) for the qualitative type-specific identification of human papillomavirus. It has a sensitivity of 98%. PapilloCheck® is a test probe which is utilised in conjunction with a DNA array analysed in a computer-controlled, high-resolution optical microarray scanner. This ensures reproducible and objective results of samples. With this test, a total of 6 low- and 18 high-risk HPV subtypes can be detected. (95)

1.5 Anatomy of the tongue, oropharynx and larynx

Tongue
The tongue is a muscular organ that forms part of the floor of the oral cavity. The tongue is essential in functions such as taste, deglutition (swallowing), articulation (speech), mastication (chewing) and oral cleaning. The lingual septum divides the tongue into the left and right side. The tongue is furthermore divided into anterior (oral/mobile tongue) and posterior (base of tongue) parts. The anterior is the mobile part of the tongue that is located in the oral cavity. This constitutes two thirds of the tongue, which is further divided by the terminal sulcus. The posterior part of the tongue is located in the oropharynx. The foramen cecum is a remnant of the thyroglossal duct, and is located at the tip of terminal sulcus. The base of tongue contains the lingual tonsils, the inferior most portion of Waldeyer's ring.

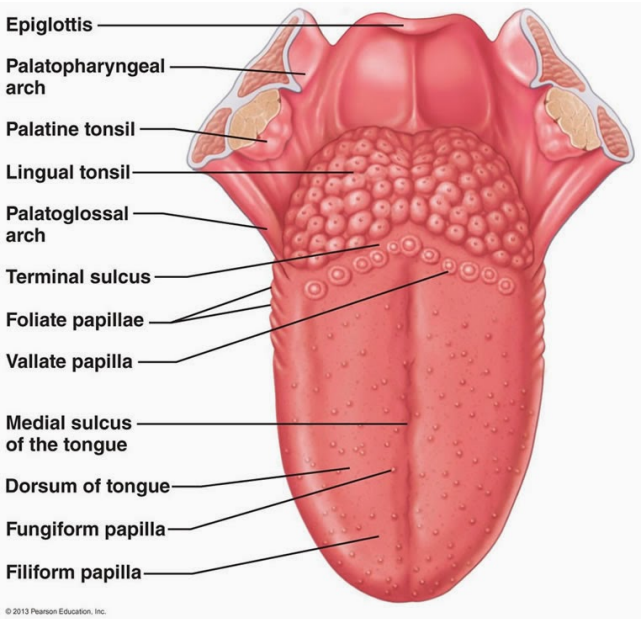

Figure 3. Anatomy of the dorsal tongue (open knowledge project by Cancer Research UK).

The tongue consists of eight muscles, classified as either intrinsic or extrinsic. The four intrinsic muscles (superior and inferior longitudinal muscles, vertical and transverse muscles) change the shape of the tongue and are not attached to bone. The four extrinsic muscles (genioglossus, hyoglossus, styloglussus, palatoglossus) act to change the position of the tongue, and are anchored to bone. The lingual papillae cover the surface of the body of the tongue and are projections of the lamina propria covered with epithelium. There are 4 types of lingual papillae: vallate (circumvallate), foliate, filiform, and fungiform. The fungiform, foliate, and circumvallate papillae are known as the gustatory papillae. They contain taste buds and work as sensory organs.

The main artery that supplies blood to the tongue is the lingual artery, a branch of the external carotid, along with accompanying lingual veins. The different veins of the tongue drain into the internal jugular vein. Concerning sensory innervation, the anterior two thirds of the tongue are supplied by (1) the lingual nerve (of the mandibular nerve) for general sensation and by (2) the chorda tympani (a branch of the facial nerve that runs in the lingual nerve) for taste. The glossopharyngeal nerve supplies the posterior third of the tongue, including the vallate papillae for both general sensation and taste.

Oropharynx

The *oropharynx* is the middle part of the pharynx that is localized posteriorly of the oral cavity, extending from the uvula to the level of the hyoid bone. It consists of the

base of tongue and the epiglottic vallecula inferiorly, the inferior surface of the soft palate and uvula superiorly, the palatine tonsils, tonsillar fossa and tonsillar pillars laterally and the posterior pharyngeal wall posteriorly. It constitutes both part of the digestive system and the conducting zone of the respiratory system. It is lined by non-keratinized squamous stratified epithelium.

The *palatine tonsils* (frequently referred to as the "tonsils") are located in the lateral walls of the oropharynx, between the palatoglossal and palatopharyngeal arches. The tonsils contain lymphoid tissue, and are part of the Waldeyer's tonsillar ring. This also includes the adenoid tonsil, the lingual tonsil and the lymphoid tissue in the posterior pharyngeal wall. The tonsils are largest relative to the diameter of the throat in young children, where they sometimes cause upper airway obstruction. They reach their largest size near puberty. In adults, the tonsillar tissue gradually undergoes atrophy.

The tonsils are lined with non-keratinized squamous stratified epithelium, and contain four lymphoid compartments: the crypt epithelium, the follicular germinal centre with the mantle zone and the interfollicular area. These all participate in the immune response.

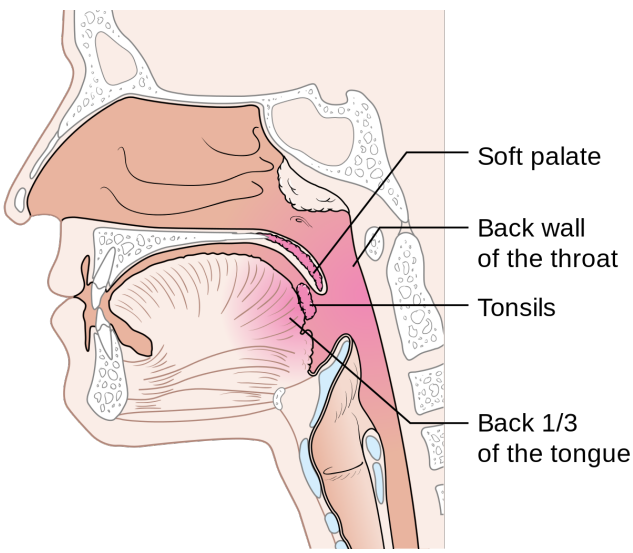

Figure 4. The parts of the oropharynx are presented. This image has been released as part of an open knowledge project by Cancer Research UK.

Larynx

The larynx is located in the upper part of the airway, and it holds a strategic position in the crossover between the respiratory and gastrointestinal tracts. It combines three

functions: 1) respiration (open airway to the lungs), 2) sound and speech generation, and 3) protective function, by preventing foreign objects, fluids and foods, from entering the trachea, bronchial tree and lungs. The larynx is subdivided anatomically into a supraglottic, glottic, and subglottic compartment (Figure 5). The supraglottic larynx encompasses the epiglottis, the false vocal cords, the arytenoids and the ventricles. The glottis consists of the true vocal cords including the anterior and posterior commissures, and it extends approximately one cm below the vocal cord into the paraepiglottic space. The subglottic larynx starts below the glottis and includes the cricoid cartilage. Below the cricoid is a transit zone towards the trachea with its first tracheal ring. The larynx is lined by squamous epithelium.

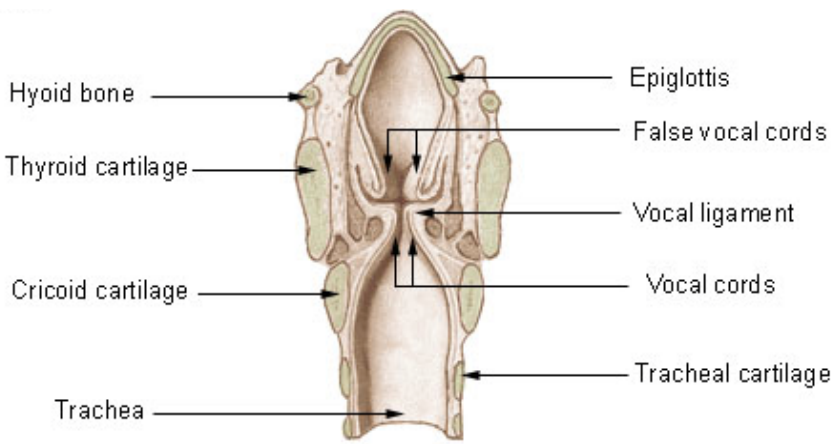

Figure 5. The anatomy of larynx. This file has been identified as being free of known restrictions under copyright law, including all related and neighbouring rights.

The muscles of the larynx are divided into intrinsic and extrinsic muscles. The intrinsic laryngeal muscles are responsible for controlling sound production, while the extrinsic muscles support and position the larynx within the trachea. Innervation of the larynx is provided by branches of the vagus nerve on each side of the neck. The external branch of the superior laryngeal nerve innervates the cricothyroid muscle and provides sensory innervation to the glottis and laryngeal vestibule. On the other hand, the inferior (recurrent) laryngeal nerve provides motor innervation to all other muscles and sensory innervation to the subglottic area.

1.6 Voice evaluation and voice production

Recurrent respiratory papillomatosis (RRP) is associated with a high grade of voice handicap due to first, the papilloma lesions affecting the voice and resonance source in the larynx, and second, the recurrent surgical outcomes. Surgical intervention aims at radically removing the lesion with optimal organ preservation. However, this

does not always result in voice function preservation. The pathophysiological vocal fold effects of the lesion itself, as well as the postoperative consequences, can alter local microcirculation, and lead to fibrosis, chronic inflammation as well as oedema of the cords and its surrounding tissues. (96, 97)

Voice can be measured using patient experiences, acoustics, phonetograms, electroglottography, electromyography and perceptual analysis of the voice. In this thesis (Study I), the voice was evaluated using patient-reported outcomes in order to assess the impact on life and voice in a RRP population and encompass any report stemming from the patients themselves regarding his or her condition. In this way, the reporting should be free from interpretation by clinicians.

Voice production involves a three-step process. First, air pressure is transmitted from the lungs towards the vocal cords by the coordinated action of the diaphragm, abdominal muscles, chest muscles and rib cage. Second, the air pressure change sets the vocal cords into an oscillatory motion when there are pressure differences above and below the vocal cords. The pressure conditions make the membranous (phonatory) portion of the cords move in a semi-cyclic vibration pattern. The vibrations are age- and gender dependent. Third, the sounds generated by the vocal cords are filtered through the resonance tube and come out of the mouth as voice that can be perceptually described from different observation dimensions as distinctive qualities of each person's voice.

1.7 Recurrent respiratory papillomatosis (RRP)

Recurrent respiratory papillomatosis (RRP) is a neoplastic disease of infectious origin that is caused by specific genotypes (6 and 11) of human papillomavirus (HPV). RRP is characterized by benign, wart-like lesions that cause hoarseness and airway obstruction. RRP accounts for extensive morbidity due to its influence on breathing, voice and recurrence in surgical sessions. (98)

1.7.1 Epidemiology of RRP

Two types of RRP are recognized: juvenile-onset RRP (JoRRP) and adult-onset (AoRRP). There is some confusion in reporting of these since the age proposed as cut-off points for the JoRRP and AoRRP vary between 12 and 20 yrs. The most frequently used cut-off point is the age of 18 years, that is, JoRRP includes patients<18 years while AoRRP patients≥18 years. (99) Only a few population-based studies are published. The overall incidence rates of JoRRP and AoRRP are suggested to be somewhere between 0,17 and 0,54 per 100 000. (100) RRP is the most common benign neoplasm of the larynx in children, and the second most frequent cause of childhood hoarseness. (101) In contrast to AoRRP, JoRRP seems to have a gender-neutral distribution. (102) HPV is often present in healthy, unaffected vocal cord mucosa, and it is not possible to distinguish infected from

non-infected epithelial cells. (103) In AoRRP, the peak incidence is around the age of 30 years, with a predominance of males. (103, 104) The cause of the male predilection is unknown, however also reflected in HPV-related HNSCC incidence, (62) suggesting growing evidence for viral transmission during oral sex and kissing. (105, 106)

Another possible explanation of AoRRP could be a reactivation of a latent HPV infection acquired in childhood. JoRRP tends to be more aggressive and is most likely caused by vertical (intrapartum or perinatal) transmission between mother and child for children delivered vaginally, especially if the labor was prolonged. (107) Caesarean section has been associated with reduced risk in epidemiological studies. (108) However, there is no clear-cut evidence for the protection against RRP using caesarean section when the mother has genital warts. It has been suggested that there is a 200-fold increased risk of JoRRP when the mother has a history of genital warts during pregnancy. (109) Despite the fact that HPV DNA was recovered from 30% of the nasopharyngeal secretions of newborn children exposed to HPV perinatally, only a small proportion of those children developed RRP. (110) This indicates the likelihood of necessary co-factors for developing JoRRP.

Although the disease can occur in almost any part of the respiratory tract, it affects the larynx and the voice source in the majority of infections. (111) Despite the benign nature of RRP, there is significant morbidity due to the multiple recurrences of the lesions. The disease burden is high, and numerous hospital admissions and surgical procedures are required to improve voice quality, and in serious cases to keep the airway patent. RRP tends to affect patients from families with low socioeconomic status at a higher rate. (112)

1.7.2 Clinical features of RRP

The main clinical characteristics of the disease are hoarseness, and in advanced cases even respiratory distress. (113) Less common presenting symptoms include dysphagia, recurrent pneumonias, failure to thrive, and chronic cough. (98)
The clinical course of RRP is highly variable. Some RRP patients experience a spontaneous remission after a relatively short period of symptoms, while others require repetitive surgical sessions due to the aggressive type of the disease and sometimes tracheotomy. (114) Notably, HPV DNA (both low- and high-risk genotypes) has been detected in 7-58 % in laryngeal carcinomas without pre-existing clinical RRP. (115) Previous radiotherapy, (116) high-risk HPV genotypes, and smoking (117) are co-factors associated with higher risk for malignant transformation. The aggressiveness of RRP is associated with low age and HPV11 genotypes. (111, 117)

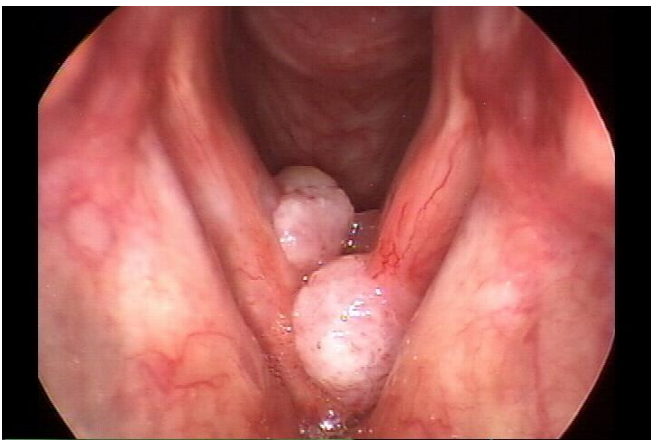

Figure 6. Bilateral RRP lesions. Reproduced by kind permission of Dr. Richard J. Vivero.

1.7.3 Surgical and adjuvant treatment of RRP

Surgical treatment

At present, there is no definite "cure" for RRP. Currently available treatments aim at reducing symptoms through surgical removal of the RRP lesions and preserving the normal structures and function of the larynx.

High-frequency jet ventilation (HFJV) is the preferred method of ventilation during surgery, since it provides full access to the larynx and thus improves the operative field for radical surgery with preserved functionality. (118) It has been speculated that distal spread of RRP can be facilitated by open ventilation systems such as HFJV with CO2 laser. (119) It may be argued that optimal access to the surgical area improves radical scope of the resection, reduces postoperative complications such as scarring, and promotes fewer numbers of surgical sessions requiring general anaesthesia. At the same time, HFJV RRP surgery should be performed in operating rooms with high air exchange/minute, optimized local air extraction, and capture (and eliminated) exhaled patient air, to protect surgery and operating room staff and avoid transmission of virus, since we don't know how the virus is transmitted.

Surgical excision is usually accomplished using the carbon dioxide (CO2) laser, which has replaced cold instruments. This method can accurately vaporize the lesions with minimal bleeding. The CO2 laser must be managed carefully. (98) An emerging technique is debulking of the lesions using microdebrider ("shaver") with improved outcomes for voice quality and reduced operation time, less tissue injury, and an important cost benefit. (120, 121) Attempts have also been made to surgically remove the RRP lesions with radiofrequency cold ablation (coblation), with reports of longer symptom-free periods between surgeries. (122) It is generally recommended to leave residual papilloma tissue during surgical removal of an

extensive disease, if it is judged that removing all papilloma will jeopardize postoperative functionality, due to risk for glottis scarring and/or web formation. (98)

Tracheotomy is the last surgical measure in aggressive RRP cases that cause critical airway embarrassment. Despite this, some authors recommend avoiding tracheotomy unless absolutely necessary since it has been associated with distal spread of the disease to the trachea and lungs. (123) If the procedure becomes unavoidable, decannulation should be attempted as soon as possible, since the disease can be managed by other techniques.

Adjuvant medical treatment

Since surgery is not curative, numerous therapies have been used in combination with surgical debulking, in an effort to reduce the RRP lesions. Adjuvant treatment options have applied in well-defined subgroups of RRP, and more specifically, in the case of frequent recurrence of the disease. (124)

Cidofovir (Vestide, Heritage Pharmaceutical, Edison, NJ, USA)

Cidofovir is an antiviral agent first used in 1995 for adjuvant treatment of severe RRP, which has since become one of the mainstays of adjuvant therapy. (46, 125, 126) The use of Cidofovir for RRP is off-label, since the compound is approved by the US Food and Drug administration (FDA) only for treatment of cytomegalovirus (CMV) retinitis in patients with AIDS without renal dysfunction. Since 1995, several case series have been published reporting beneficial effects of Cidofovir in severe RRP. Large, randomized, placebo–controlled, prospective trials the last five years have not been able to confirm the previous carcinogenic concern. (127, 128) However, more high quality data, especially using randomized controlled trials, are still required to provide evidence of the efficacy of Cidofovir.

α-Interferon

α-Interferon has been used since 1988. The medicine significantly reduces the severity of the disease, however at discontinuation, disease severity rapidly returns to pre-treatment levels. (129) Systemic α-interferon is less popular due to its unfavourable side effects.

Bevacizumab (Avastin)

Prospective investigations and retrospective cohort studies, as well as case series have been conducted both in JoRRP and AoRRP. Avastin was administrated intralesionally as an adjunct to angiolytic KTP laser treatment. Promising results are reported regarding efficacy without significant complications. (130) Long-term results and larger blinded randomized studies are warranted.

Indole-3-carbinol

Indole-3-carbinol, a dietary supplement, has been suggested in the treatment of RRP and has showed promising results when used in animal experiments (131) or small clinical groups. (132) Previous reports have claimed successful treatment outcomes. (132)

HPV vaccine
The HPV vaccine procedure is described in section1.3. A two-dose schedule is recommended and applied in Sweden since 2015 for girls aged 9-13 years. The currently used Gardasil 9® has the ability to protect against 90% of cancers of the cervix, vulva, vagina, and anus. The use of Gardasil® in therapy has been presented with promising results in selected cases. (133)

Treatments with mumps vaccine, (134) ribavirin, (135) and photodynamic therapy (PDT) (136) have also been in use. Nevertheless, longer follow-up and trials in larger groups are warranted. Overall, viral persistence seems to occur following all these adjuvant treatment methods, as with surgery.

1.7.4 Prevention with HPV vaccines
There is no proven efficacy in the prevention of recurrent respiratory papillomatosis. (90, 91) However, as with anogenital warts, the low-risk genotypes HPV6 and 11 account for the majority of RRP cases. It is therefore conceivable that the 9-valent recombinant HPV vaccine will have impact on the future incidence of RRP. (137) There is a clear predominance of men in RRP as well as oropharyngeal cancer urging the gender-neutral vaccination strategy. The expected vaccination-induced reduction of anogenital HPV infections might additionally decrease the incidence of RRP in future generations. (138, 139) This preventive and perhaps even therapeutic HPV approach seems to be promising, however future trials with larger material in order to test the feasibility of vaccination are warranted.

1.8 p16
p16 is a 156-amino-acid protein and it is codified by a gene localized on chromosome 9p21 within the INK4a/ARF locus. (140) It is suggested that this protein plays a significant role in cell cycle regulation by decelerating cells progression from G1 phase to S phase. It acts therefore as a tumor suppressor protein that is implicated in the prevention of malignancies. It is an inhibitor of cyclin dependent kinases such as CDK4 and CDK6 that phosphorylate retinoblastoma protein (pRB), which eventually results in progression from G1 phase to S phase. (141) The way p16 acts as a tumor suppressor is by binding to CDK4/6 and preventing its interaction with cyclin D. This leads ultimately to the inhibition of the downstream activities of transcription factors, such as E2F1, and arrests cell proliferation. (142)

Moreover, p16 is overexpressed in HPV-positive oropharyngeal carcinomas due to the degradation of pRb by the viral oncoprotein E7, which normally is a negative regulator of p16. (143) As a result, p16 overexpression correlates to HPV positivity in oropharyngeal cancer and can therefore be used as a surrogate marker for HPV positivity in these carcinomas. (43, 144) However, the correlation between p16 and HPV status in the oropharynx is complex and organ-specific. Prognostic advantage for p16-expressing oropharyngeal tumors is well-established, and could indicate a significant step in overall and disease-free survival as well as locoregional tumor control. (145) p16 is identified by immunohistochemistry.

Beside this, homozygous deletion of p16 is frequently found in oesophageal and gastric malignancies. (146) Hypermethylation of p16 is also being considered to be a potential prognostic biomarker for prostate cancer. (147)

1.9 Head and neck cancer (HNSCC)

1.9.1 Epidemiology and risk factors of HNSCC

Approximately 650 000 new cases of squamous cell carcinoma of the head and neck (HNSCC) are diagnosed each year worldwide. (148) The incidence in Sweden is approximately 1 400 new cases each year, (149) accounting for 3% of all new cancer diagnoses in the country. In general, this incidence is twice as high in men compared to women. (150). Smoking and alcohol are well-established risk factors for developing HNSCC. Other well-known factors are betel nut and tobacco chewing. (151) Viruses have also been implicated as causative factors; Epstein-Barr virus (EBV) has been associated with nasopharyngeal cancer (152) and HPV with oropharyngeal cancer. (153) High lifetime number of vaginal and oral sex partners, high-risk HPV and seropositivity for HPV16 have been reported in the context of development of oropharyngeal cancer. (106)

1.9.2 TNM classification and staging of oropharyngeal cancer

The most widely used classification system for head and neck cancer is the TNM system developed by the international Union Against Cancer. (154) The classification is based in the size and extension of the tumor (T), presence, size and localization of regional lymph node metastasis (N), and presence of distant metastasis (M). Staging is the process of classifying a primary tumor depending on the extent of the cancer, including the presence or absence of metastases. It aids in treatment planning, prognosis determination and communication between healthcare centres.

Table 1. Primary tumor (T) (oropharynx).

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

Table 2. Regional lymph nodes (N).

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

Table 3. Distant metastasis (M).

M0	No distant metastasis
M1	Distant metastasis

Table 4. Anatomic stage/prognostic groups.

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

TAny: any of the available classifications of primary tumor size.

NAny: any of the available classifications regarding the location of lymph node metastasis.

1.9.3 Treatment of HNSCC

The oncologic treatment is primarily aimed at survival. Due to the important communicative and digestive aspects of the affected organs, functional preservation is increasingly desirable. Surgery and radiotherapy (RT) were considered to be the only curative treatment of patients with squamous carcinoma of the head and neck back in the 1960-1980´s. Today, surgery may be used as single therapy in selected low-stage disease. RT is the mainstay of treatment, and it can be used as single modality treatment or combined treatment. In the majority of cases, a combination of treatments is needed for optimal results. The resection of locally advanced tumors has been made easier due to microsurgical free tissue transfer for reconstruction of surgical defects. Chemotherapy can be administrated as *induction therapy*, that is, given prior to radiotherapy, or *concurrent therapy,* administrated simultaneously as radiotherapy, (155) or as *adjuvant* chemotherapy after surgical resection and in cases of residual disease. (156, 157) The benefits of chemotherapy must be balanced with adverse effects due to its increased toxicity, especially among patients with medical comorbidities and reduced performance status. Organ preservation does not equal functional preservation, making the choice of therapy in specific combinations challenging. The combination of surgery and radiotherapy is more commonly used; RT can be given either pre-operatively or post-operatively depending on the treatment protocols of each hospital in different countries.

Radiotherapy (RT) is an integral part of primary or adjuvant treatment for HNSCC. It results in high tumor control and cure rates for early stage HNSCC including glottic, base of tongue, and tonsillar cancer. Prior to radiotherapy start, a plastic mask is fitted to the patient's face using a vacuum cushion, aiming to keep the head in the same position for each treatment session. A computed tomography (CT) scan is performed in the RT treatment position. A dosimetric plan is established, and treatment can commence. Treatment of HNSCC employs fractionated radiotherapy protocols. This implies that the total radiation dose is subdivided into smaller doses given over a certain time.

1.9.4 Prognosis of HNSCC

Survival rates are dependent on stage and site of tumor. Cure rates decrease in locally advanced cases and regional node involvement. The overall five-year survival for all stages of HNSCC is estimated to be between 35% to 50% due to late disease presentation. Up to 50% of head and neck cancer patients present with advanced disease. (158) This 5-year mortality rate has not improved significantly in the last few decades, despite advances in treatment modalities, and reduced exposure to traditional risk factors. (159) Figures 7 and 8 depict the percent of cases and 5-year relative survival by stage at diagnosis according to SEER (Surveillance, Epidemiology, and End Results Program) of the American National Cancer Institute, 2005-2011.

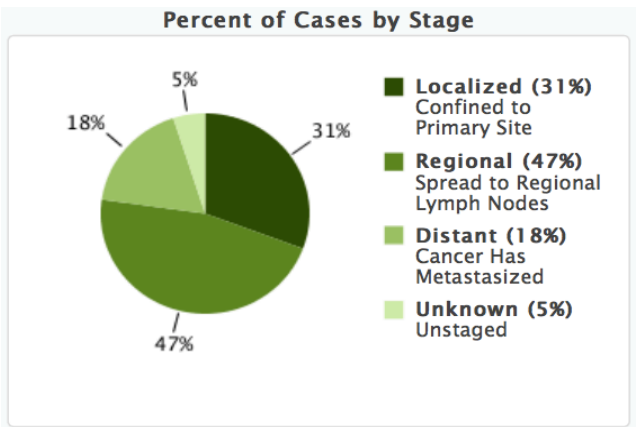

Figure 7. Percent of cases at diagnosis: oral cavity and pharynx cancer.

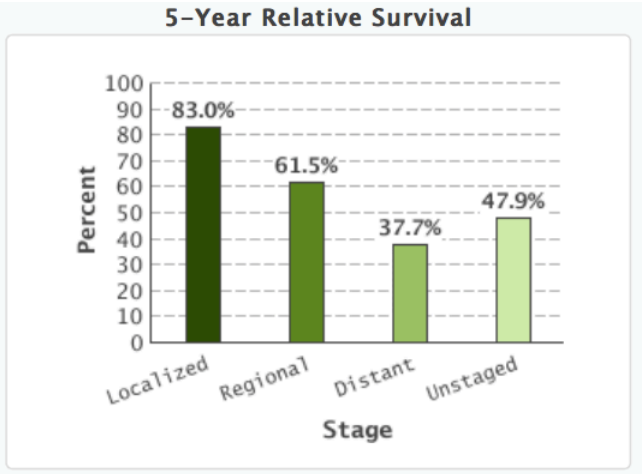

Figure 8. Percent of 5-year relative survival by stage at diagnosis: oral cavity and pharynx cancer.

1.10 Tonsillar cancer

HPV-positive tonsillar cancer is considered to be a different tumor entity compared to HPV-negative carcinomas. The former arise in the tonsillar crypts while the latter originates from the epithelium of the tonsillar surface. (160) As previously reported, many of the HPV-positive tonsillar cancer cases lack the traditional risk factors associated to the disease. The patients tend to be younger and generally have a better performance status. (159) Most HPV-positive tonsillar cancers are associated with the high-risk HPV16 (in approximately 95% of the HPV-positive cases), (161) while other subtypes (31, 33 and 35) are relatively infrequent. Overall, patients affected by HPV-positive tonsillar cancer have a better prognosis and improved survival, regardless of treatment strategy when compared to HPV-negative cases. (162-164) Despite this, the biological and molecular mechanisms behind the difference in survival have yet to be revealed. Treatment modalities have so far not been adjusted to the presence or absence of HPV. Previous studies have established an inverse relationship between HPV tumor status and the presence of p53 mutations in head and neck cancer. (165)

1.10.1 Epidemiology and risk factors

The incidence of tonsillar cancer appears to be increasing over time during recent years in several western countries, preferentially in men. (166-168) Tonsillar cancer is the most common form of oropharyngeal cancer, (150) which develops in the base of the tongue and palatine tonsils, posterior pharyngeal wall, and soft palate. Heavy tobacco smoking and alcohol consumption are known traditional predisposing factors and seem to act synergistically in the development of the disease. (169) Despite a decline in smoking and alcohol abuse (170) during the last years, the

incidence of tonsillar cancer appears to be increasing worldwide. HPV has been regarded an important aetiological agent. (166, 167, 171, 172) Recent studies report a high prevalence of HPV and EBV co-infection in base of tongue and tonsillar cancers. (173)

Demographically, patients with HPV-positive tonsillar malignancies are often white males, with high socioeconomic status, non-drinkers and non-smokers. (106, 159, 174) Changes in sexual behaviour and increased practice of oral sex could account for some of the increased incidence of HPV-associated cancer in younger generations. (167) Other factors that could be implicated are higher number of lifetime sex partners and earlier age at sexual debut. (106, 175) In addition to this, increased risk for tonsillar cancer among women with cervical lesions and husbands of women with cervical dysplasia or cancer has been identified. (176)

1.10.2 Clinical features of tonsillar cancer

Squamous cell carcinoma of the head and neck (HNSCC) is a collective term for malignant disease in the ear, nose, and throat region. It is the sixth most common malignancy, with a significantly varying incidence in different world regions. (148) Typical symptoms are unilateral sore throat and earache, swallowing difficulties, neck lump that turns to be a nodal metastasis, unexplained weight loss, and fatigue. Unfortunately, many patients are asymptomatic initially and therefore, diagnosed in later stages of the disease. It has been suggested that HPV-positive tonsillar carcinomas should be considered different tumor entities from HPV-negative tonsillar tumors based on epidemiology, genetics, response to therapy, and prognosis. (62) Therefore, patients could benefit from less aggressive treatment. (160, 177)

1.10.3 Treatment of tonsillar cancer

Adequate and proper investigation is necessary for planning optimal treatment with curative intentions, organ as well as functional preservation. (178) Current recommended management of patients with HPV-induced tonsillar malignancies do not differ from those who are HPV-negative. (160) As standard of care, radiotherapy is considered to be the best and most effective treatment option today, combined with concurrent chemotherapy in cases of advanced (stages III and IV) tonsillar cancer. Clinical trials evaluating new surgical approaches and especially, transoral robotic surgery (TORS), are now underway. (179, 180) Unresectable disease is usually treated with concurrent chemoradiotherapy. This has been shown to have superior survival rates compared to monotherapy with radiation alone. (181-183) The mortality rate is high due to advanced disease at diagnosis, the need for aggressive treatment strategy, high risk for locoregional recurrences, and co-morbidities. The single most debilitating side effect of RT is oropharyngeal mucositis with subsequent sore throat and mouth sores. Long-term sequelae include

dysphagia, xerostomia, weakness and fatigue, candidiasis and osteoradionecrosis. (184, 185)

Surgery vs. radiotherapy during the last decades
Surgical resection of tonsillar tumors is technically challenging, even when properly performed, since it takes place in a complex anatomical area. Currently, surgical resection is reserved as 'salvage surgery' in cases of residual or recurrent tumor after oncological treatment. Recent results using transoral robotic surgery (TORS) as single modality treatment have shown promising results, especially for small tumors of the tonsillar fossa that do not extend toward the lateral pharyngeal wall or the base of the tongue. (179, 180) Studies have reported excellent locoregional control rates, ranging from 80% to 90%. (179, 186) Neck dissection is performed in case of nodal cervical metastases that are either extensive or do not respond to radiotherapy.

1.10.4 Prognosis of tonsillar cancer

The overall 5-year survival rate for SCC tonsillar cancer is related to the stage of the disease; approximately 90% for stage I tumors, whereas patients with stage IV tonsillar cancer have a survival rate of less than 20%. (187, 188) It has been reported that non-smoking patients with HPV-positive tumors have a better prognosis than smokers, (159) and HPV-positive males have improved survival rates over HPV-positive females. (189) Studies indicate that p16 can be used alone as a prognostic test for tonsillar cancer survival but combined p16 and HPV testing seems to be superior in predicting survival. (190, 191)

As mentioned before, patients with HPV-associated tumors in the tonsillar region are younger and exhibit better prognoses than their HPV-negative counterparts, regardless of treatment modality. (163, 164, 169) This poses the question of whether the aggressive chemoradiotherapy and surgery applied to patients with HPV-positive tumors could be de-escalated in an effort to reduce toxicity, and by doing this improve the long-term quality of life while maintaining treatment efficacy. The suggested treatment modification due to presence or absence of HPV in malignancies might be achieved by the following: 1) reducing the total dose of radiotherapy, 2) using radiotherapy alone without chemotherapy, 3) using radiotherapy with less toxic chemotherapeutic agents (for example, EGFR inhibitors), and 4) using TORS as single-modality treatment with selective neck-dissection when appropriate. (177)

The reasons for the better response in HPV-positive cases are so far unknown. Some studies might suggest immunological factors related to HPV infection (192) while others focus on the inverse relationship between HPV status in the tumors and the presence of mutations in HNSCC (intact p53-mediated apoptotic response). (165)

1.10.5 HPV vaccines

The HPV vaccine procedure is described in section1.3. Gardasil 9® is a 9-valent recombinant vaccine against human papillomavirus and a further development of Gardasil®. The vaccine has the ability to protect against 90% of cancers of the cervix, vulva, vagina, and anus. If 90% of females were vaccinated, this could theoretically reduce the number of HPV associated oropharyngeal cancer in men by 66%. (89) Thus, the current vaccination of females positively affects the rates of oral HPV infection in the males. However, it would still be an unsatisfied HPV-related increase in oropharyngeal cancer in men. (89) The numbers of HPV infections in the oropharynx and subsequently, HPV-associated tonsillar cancer cases, are expected to outstrip the rate of cervical malignancies by 2020. This highlights the importance of performing a gender-neutral vaccination strategy in the future. (193)

1.11 Tongue cancer (TSCC)

The tongue is the most common site for cancer presentation in the oral cavity worldwide, (194) and constitutes a major public health problem in a number of countries, causing significant morbidity and mortality. Malignant tumors of the mobile (oral) tongue constitute a challenge due to their unique behaviour, requiring aggressive treatment to minimize the risk of locoregional spread.

1.11.1 Epidemiology of TSCC

Squamous cell carcinoma of the mobile tongue (TSCC) is considered to be one of the most commonly presented head and neck cancers, with an estimated 12 770 new cases in the USA in 2012. (194) In Sweden, tongue cancer constitutes about 12% of all HNSCCs. (195) Increasing incidence trends over the last decades have been reported, (196) particularly in young white females aged 25-44, (195) Afro-Americans, Hispanics and Asians. (197) According to Scandinavian tumor registries, the general incidence of oral cancer SCC increased 5-fold among young men and 6-fold among young women (years 1960-1994) compared to just a 2-fold increase in older patients. (195) In general, malignant tumors of the anterior two-thirds of the tongue are detected earlier than cancers of the oropharyngeal posterior one-third; the mobile tongue cancers are usually better differentiated. (198)

The most commonly named etiological factors for cancer of the mobile tongue are tobacco and alcohol abuse. (199) However, these particular risk factors are not commonly present among the majority of the young patients. Additional risk factors have been discussed including nutritional deficiencies, poor dentition, viruses, (200, 201) and genetic factors. (202) Additionally, the unique gender- and age-specific incidence trends suggest a possible role for bacterial infections, lifestyle, and environmental factors. (197) Squamous cell carcinomas are estimated to constitute about 90-92% of all tongue carcinomas. The remaining tumors are

adenocarcinomas, lymphomas and sarcomas, and they differ from the former in terms of aetiology and carcinogenesis.

1.11.2 Clinical features of TSCC

Leukoplakia, erythroplakia, and chronic glossitis are lesions that appear in the oral cavity with the potential for malignant transformation. (203) Symptoms are usually present in tumors larger than 1 cm, and more commonly involve swallowing, articulation difficulties, articulation disabilities and pain, when the tumor involves the lingual nerve. In such cases, referred pain to the ipsilateral ear may be present. Additionally, dysphagia may lead to aspiration as well as malnutrition.

1.11.3 Treatment of TSCC

In general, superficial lesions are best treated with curative intention and single-modality therapy with surgical excision. Multiple modalities (combination of surgery and radiotherapy) are required for larger tumors and cervical metastasis. As inadequate excision of the primary tumor is considered to be the most common cause of tumor-related death, efforts should be made to obtain clear and wide margins of resection during surgery.

The two main techniques of radiotherapy that are currently being used are external beam radiotherapy (204) and brachytherapy. (205) The role of chemotherapy in the treatment of mobile tongue cancer still remains unclear. Tumors of early stage are not treated with chemotherapy. Advanced primary lesions or the presence of distant metastases and generally poor prognosis constitute an indication for chemotherapy treatment. (206)

The treatment of choice of TSCC aims primarily at survival, but the digestive aspects, with high risk of aspiration, make an organ preservation treatment choice desirable especially in patients with poor general health status. The healthy tissue is highly affected in the surgical, chemotherapy, and radiation process, with major side effects that have to be manageable for the patient. Once radiation has been used in the affected tumor area, the possibility to use it again in case of disease recurrence is reduced.

1.11.4 Prognosis of TSCC

Several reports describe squamous cell carcinoma of the mobile tongue as an aggressive disease among young adults. Some of these reports have demonstrated lower survival rates for young patients compared to older individuals. (207, 208) At the same time, a smaller number of studies indicate better survival rates among younger patients. (209) The overall 5-year survival among patients with TSCC has been reported as between 37% and 85%. (210, 211) The survival rate is highly dependent on nodal status at the time of diagnosis with 50% 5–year overall survival

in patients with N0 neck compared with 11% in patients with nodal metastasis. (212) Tumor thickness is also an important factor for the prognosis, and has been positively correlated to nodal disease. (213)

Recurrence rates have been estimated between 27 and 40%; (211, 214) significant prognostic factors for local recurrence include T-stage, histopathological grade, time interval between surgical treatment and RT, age, sex and total dose of radiation. (215-218) It is generally accepted that patients should undergo monitoring for a minimum period of at least 5 years after treatment because of the high recurrence rates at both primary and neck sites as well as the increased frequency of distant metastasis and second primary malignancies.

2. Purpose and aims

2.1 Overall purpose

The overall purpose of this thesis was to describe the human papillomavirus (HPV) in recurrent respiratory papillomatosis, tonsillar cancer and tongue cancer.

2.2 Specific aims

- To measure the effects of RRP on voice and quality of life, and to assess the relation of early manifestations of the disease to long-term morbidity.
- To assess the incidence of tonsillar cancer in northern Sweden and study the impact of HPV virus and its surrogate marker p16 in tonsillar cancer.
- To determine clinical characteristics and possible predictor factors affecting the therapeutic needs of RRP patients in northern Sweden, and to identify a potentially high-risk RRP group.
- To assess the clinical and prognostic importance of HPV, p16 and HPV receptor syndecan-1 in mobile tongue cancer and tonsillar cancer.

3. Patients and methods

3.1 Study design

All studies employed quantitative methodology; data was retrieved from population-based descriptive prospective longitudinal trials (Studies I and III) and a retrospective observational cohort study (Studies II and IV). Table 5 summarizes the study design, population and sample size of included papers.

Table 5. Study design.

	Study I	**Study II**	**Study III**	**Study IV**
Design	Prospective	Retrospective	Prospective	Retrospective
Subjects *(n)*	27	214	48	109
Male/female	17/10	155/59	34/14	54/55
Time interval	2004-2012	1990-2013	2004-2014	1997-2012

All studies included in this thesis were approved by the Regional Ethical Review Board in Umeå (Dnr. No2012 276 32M, 2010 277 31, 08-003M and 03-201). To access the accurate retrospective paraffin-embedded samples from the BioBank North, we used data from the Swedish Cancer Registry database. The application was approved by the Biobank North (472-13-08 in 2013-03-26). All patients in the prospective studies gave their informed consent after receiving information on the details of the study according to the Helsinki declaration. In Study II, a detailed outline of the patients included and excluded is provided in Figure 6. In Study III, 21 consecutive RRP patients were added to the 27 patients from Study I. All 48 patients were assessed for eligibility, fulfilling the in-, and exclusion criteria (Table 6), none discontinued participation.

Study I

This study was a prospective questionnaire-based cohort study that included 27 consecutive, non-smoking patients (age 21–71 years, median 47 years) that were presented, diagnosed and treated for RRP at the Department of Otorhino-laryngology, University Hospital of Umeå, Sweden, between 2004 and 2012. In-, and exclusion criteria for participants in Studies I and III are provided in Table 6.

Preoperatively, the larynx was examined using transnasal flexible endoscopy or flexible videoendoscopy with high definition technique, stroboscopy and narrow band imaging (NBI). The purpose was to visualize the RRP lesions and assess the voice source function. Intraoperative biopsies were obtained for histopathological studies and HPV analysis. An outpatient visit was planned within 8 weeks after surgery and the patients received the questionnaires VHI and SF-36 within 3 months

after the last treatment session.

Table 6. In-, and exclusion criteria for participants in Studies I and III.

Inclusion criteria	Exclusion criteria
• cognitive abilities • adequate Swedish language competency enabling the patient to independently answer the questionnaires	• previous tracheotomy • vaccination with HPV vaccines • treatment with adjuvant therapies (a-interferon, indole-3-carbinol, cidofovir) • previous treatment of the disease with radiofrequency coblation or microdebridement during the last 10 years • no smoking • no occurrence of allergy, gastroesophageal reflux disease (GERD) and asthma • no prematures in juvenile onset (JoRRP) • non responders of the questionnaire, n=6

Study II
This is a retrospective observational study, which included all patients diagnosed with tonsillar cancer during 1990-2013 at the University Hospital of Umeå. Information was extracted by the Swedish Cancer Registry database in order to identify cases of tonsillar cancer. 65 tonsillar cancer biopsies obtained between 2000 and 2012 were analysed. The ICD-7 code used for tonsillar cancer was 145.0. A detailed outline of the eligible patients is provided in Figure 7.

Pre-treatment tumor samples were collected by biopsy or surgical resection and paraffin-embedded tumor blocks were retrieved from the archives of the Department of Laboratory Medicine/Pathology at the University Hospital of Umeå.

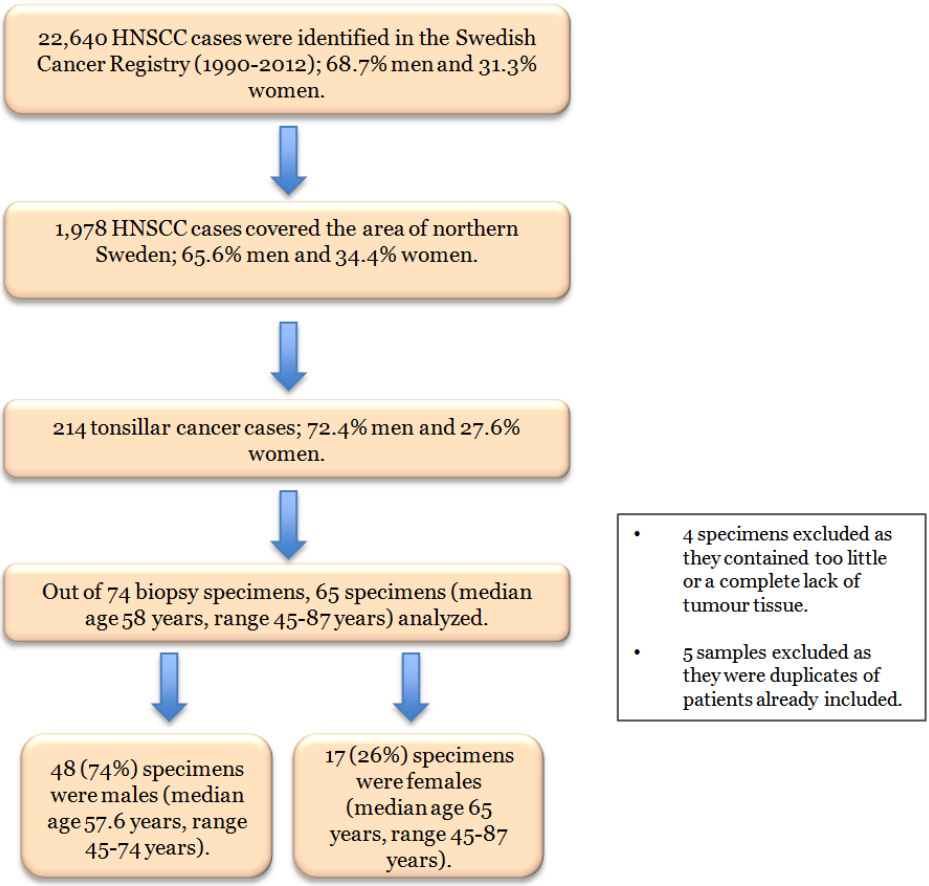

Figure 7. Flowchart of included and excluded patients in Study II.

Study III

This prospective cohort study included all 48 patients diagnosed or treated with RRP at the University Hospital of Umeå, between 2004 and 2014. We used the same in-, and exclusion criteria (Table 6), preoperative examination and biopsy procedures as in Study I. The 48 included patients were categorized for age, disease duration, onset (JoRRP or AoRRP), profile of disease development, and number of surgical sessions in relation to disease duration, localization of papilloma, gender and HPV subtypes. According to the frequency of operations, patients were divided into two groups: low-frequency (LF, <1 surgical treatment/year) and high-frequency (HF, ≥1 surgical treatment session/year) groups. Surgery was performed using CO2 laser. All patients were treated by the same surgeon and were evaluated approximately 8 weeks postoperatively and as needed afterwards.

Study IV

The study included formalin-fixed, paraffin-embedded samples from 109 patients

with primary TSCC; 96 of them were retrieved from the Clinical Pathology lab, Umeå University Hospital, Sweden and 13 from the Second University of Naples, Multidisciplinary Department of Medical, Surgical and Dental Specialties, Naples, Italy. Both patient groups received treatment during a 15-year period. Based on their age at diagnosis, patients were grouped into three groups: ≤40, 41-65 and >65 years. 66% were treated with preoperative radiotherapy followed by surgery while 31% received single modality treatment with surgery. The mean follow-up post-treatment was 45,5 months (range: 1-179) and survival was measured as: alive disease free, alive with disease, dead of disease, dead of other disease or dead with disease but not with oral cancer as first cause of death. 65 patients with tonsillar cancer (participants from Study II) were included in the analysis of syndecan-1 expression.

3.2 Study participants

A total of 48 patients, diagnosed or treated for RRP between 2004-2014 and 65 tonsillar cancer samples obtained between 2000-2012 were analysed at the tertiary referral centre for northern Sweden, University Hospital of Umeå. Northern Sweden was defined as the part of Sweden consisting of the counties Västerbotten, Norrbotten, Västernorrland and Jämtland, which include a total population of 882 563 (2015). In Study IV, 109 cases of TSCC available at both Clinical Pathology, Umeå University Hospital, Sweden and the Second University of Naples, Multidisciplinary Department of Medical, Surgical and Dental Specialties, Naples, were included.

3.3 Voice handicap index (VHI) and Short Form (36) Health Survey (SF-36)

As a result of the complex nature of the voice, multimodal assessment approaches are often advocated. (219) These include acoustics and perceptual measurement as well as patient-reported experiences. However, objective and subjective measures do not always agree. Patient-reported outcomes (PRO) encompass any report stemming from the patients themselves regarding his or her condition and should hence be free from the interpretation by clinicians. Observers often underestimate or make incorrect judgement of patient experiences. It can therefore be argued that the patient is the most reliable source of information for this purpose and also the freest from bias. The fact that acoustic analysis can be influenced by recording equipment, mouth-to-microphone distance, the software used for analysis as well as the choice of vowel, thereby hampering inter-study comparison, made this measurement technique less interesting. (220) We also chose not to perceptually analyse the voice due to the fact that the listener's profession can bias the perceptual measurement method. (221)

Voice handicap index (VHI)

The RRP patients underwent pre- and postoperative voice analysis. The patients

vocalized sustained /i/ and /m/ as close as possible to habitual speaking pitch and intensity. The patients received the VHI questionnaire within 3 months after having their last surgery; a reminder was dispatched after 6 months in case the questionnaire had not been returned.

The VHI questionnaire evaluates the effect of voice disorders on daily life. More specifically, it measures the patient's perception of voice quality, before and after treatment for laryngeal disorders. The survey includes and examines 30 items, grouped into three subscales related to voice disorders: physical, emotional and functional domains. (222) Every aspect or domain is represented by 10 items, formed as statements. Each of the 30 questions will elicit a response and corresponding score: 0 for 'never', 1 for 'almost never', 2 for 'sometimes', 3 for 'almost always' and 4 for 'always'.

The total obtained score (VHI_{total}) ranges from 0 to 120 points and each of the three subscales from 0 to 40 points. The higher the score, the greater negative impact on daily life due to the voice disorder. A voice handicap score from 0 to 30 is considered to be 'minimal' handicap, from 31 to 60 'moderate' handicap and from 61 to 120 points 'serious' voice handicap. A validated Swedish translation of the voice handicap index (Sw-VHI) (223) was reported in 2009 and concluded that 20 points or less in the total VHI score can be used as a normative value when the questionnaire is applied to the Swedish population. Moreover, a difference above 13 points for the VHI_{total} and more than 6 points for each subscale regarding the same individual and between two measurements, was considered to be a true change in the quality of voice experienced. (223)

Short Form (36) Health Survey (SF-36)

We used the SF-36 questionnaire as a complementary module to VHI in order to assess quality of life aspects in relation to RRP and VHI. As with the VHI survey, the SF-36 questionnaire was given to the patients approximately 3 months postoperatively with a reminder after 6 months in the case of no return. The SF-36 questionnaire is a 36-item, patient-reported survey of quality of life. (224) It consists of eight scaled scores, related to eight different domains of health-related quality of life: physical functioning, role limitations due to physical health and emotional problems, vitality (energy and fatigue), emotional well-being, social functioning, body pain and general health status. The scores in each domain are the weighted sums of the questions in their section and each patient receives a score for each domain, ranging from 0 (worst) to 100 (best). Combining selected domains generates a further two generalized subscales; the physical (PCS) and mental (MCS) component summary scores. Lower scores mean more disability. Two disadvantages with this survey that are well recognized are that the survey does not take into consideration a sleep variable and furthermore, it has a low response rate in the >65

population. (225)

The SF-36 survey was also applied in the generalized Swedish population (1995), in order to receive the corresponding normative values. (226) The disadvantage of this project is the lack of knowledge about the change over time in the SF-36 measurements.

Videoendoscopy and stroboscopy
Functional evaluation of the larynx was initially performed using the Olympus ENF P4 transnasal flexible endoscope and when updating the equipment the later recordings were performed using the Olympus ENF VH flexible videoendoscopy system. Initially the stroboscopic equipment was a Wolf type 5052 and the camera a Wolf endocam 5502. After an update of the system, the later recordings were based on the Olympus CV-170 light source system and the Olympus CLL-SI stroboscope unit. The rhino- and oropharynx and larynx were examined. The localization of the RRP lesions was carefully described and documented in the running hospital's documentation system, Picsara. In contrast to Derkay et al., (227) the goal was not to streamline the prediction of treatment intervals based on anatomical and symptom scores, but rather to identify associations of the other factors to the observations of the HPV deposit in the larynx. No Derkay score was therefore calculated.

3.4 HPV DNA extraction and PCR analysis

Biopsy specimens from RRP and tonsillar cancer cases were analysed by an experienced head and neck pathologist as a histopathological basis for the inclusion in the study. The outline of included and excluded specimens is provided in Figure 7. Seventy-four tonsillar cancer specimens were extracted from the archives of the Department of Pathology, University Hospital of Umeå (from 2000 to 2012). Four additional specimens that either contained too little, or completely lacked tumor tissue were excluded. Five additional samples were excluded, as they were duplicates of already registered patients. The RRP fresh tissue sample was kept in saline and the HPV genotypes were settled at the Clinical Microbiology Laboratory at Skåne University Hospital. The laboratory is accredited according to the ISO15189 standard for analysis of 14 oncogenic types and for HPV6 and HPV11.

3.4.1 DNA extraction in RRP lesions

A 2- to 3-mm biopsy from RRP lesion was immersed in 1 mL saline solution before extraction. The saline was removed and the biopsy was digested in 500 µL lysis buffer (10mM Tris-HCl; 10 mM NaCl; 10 mM EDTA; pH 7.8, 4% sodium dodecyl sulfate; and proteinase K [200 lg/mL, Roche]) at 37ºC overnight. Then, DNA was extracted with the help of the Total NA-kit (Roche) using MagNA Pure LC (200 µL input and 100 µL output).

3.4.2 Identification of HPV DNA in RRP lesions and tonsillar cancer samples

In Study II, all samples included were from formalin-fixed paraffin embedded (FFPE) diagnostic biopsies. DNA was extracted with QIAamp DNA FFPE Tissue kit (Qiagen, CA, USA) or QIAamp Mini kit (Qiagen) according to the manufacturer's instructions and a general HPV PCR was run with 100 ng extracted DNA from each patient with general primers GP5+/6+. (228) The process of detection of HPV DNA by PCR is mentioned in details in Study II. S14 primers were used to confirm the presence of amplifiable DNA. S14 was positive in all HPV-negative tonsillar cancer samples. In the RRP lesion biopsy, simultaneous identification of 39 genital HPV types was carried out by modified general primer polymerase chain reaction (MGP-PCR) and subsequent Luminex analysis in a 25 μL reaction containing 5 μL of extracted material. (229) The Luminex assay included probes for HPV types 6, 11, 16, 18, 26, 30, 31, 33, 35, 39, 40, 42, 43, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 67, 68 (a and b), 69, 70, 73, 74, 81, 82, 83, 86, 87, 89, 90, 91 and 114.

3.4.3 Quantitative RT-PCR in RRP lesions

Quantitative RT-PCR assay was performed using the QuantiTect Probe RT-PCR Mastermix (Qiagen, Stockholm, Sweden) and the Oligotex Direct mRNA Mini Kit, (Qiagen, Stockholm, Sweden) and PCR was performed by the use of ABI 7500. (230) The RRP biopsy sample adequacy was assessed by testing 5 μL of the sample for the human beta globin gene with a real-time polymerase chain reaction (PCR).

3.4.4 HPV16 in situ hybridisation in tongue cancer samples

A HPV16 plasmid DNA was amplified, purified and labelled by nick-translation in the presence of digoxigenin-16-dUTP, followed by purification and ethanol precipitation. After dewaxing, endogenous peroxidase activity was blocked in H_2O_2 in methanol and tissue digested with varying concentrations of proteinase K (Sigma). Hybridisation was performed overnight followed by immunohistochemical detection, with mouse anti-digoxin and avidin–biotin peroxidase complex. A positive control from cervix was included with each batch.

3.5 p16 analysis (immunochemistry)

In Studies II and IV, p16 immunochemistry was used in order to identify the relationship of the protein marker p16 to HPV virus in mobile tongue and tonsillar cancer samples. A Ventana staining machine was used for detection of p16. For this purpose, an antibody (monoclonal mouse anti-human; cat. no. sc-56330) (Santa Cruz Biotechnology, Dallas, Texas) was used and diluted 1:200. Pre-treatment of slides in Tris-EDTA pH 8.0 was performed before staining. Scoring percentage of tumor cells and staining intensity was calculated with the Quick score system. (231) The expression of p16 in tumor cells was divided in 6 categories: 1=0-4%, 2=5-19%, 3=20-39%, 4=40-59%, 5=60-79% and 6=80-100%. A scale was used for

calculating staining intensity: 0=negative, 1=weak, 2=intermediate and 3=strong staining and a quick score (range 0-18) was obtained by multiplying percentage of tumor cells expressing the protein with intensity.

3.6 Syndecan-1 analysis (immunochemistry)

Immunochemistry was additionally used for identification of the presence of HPV receptor syndecan-1 in selected cases of mobile (oral) tongue cancer and tonsillar cancer samples. The specimens were stained with an antibody detecting syndecan-1 (SP152; Abcam, Cambridge, UK) diluted 1:100, after pre-treatment in citrate buffer pH 6.0 and staining was performed in a Ventana staining machine. The intensity of staining was calculated in the same way (Quick score system) as reported before for p16 analysis. (231)

3.7 Swedish Cancer Registry and BioBank North

When retrieving cases of tonsillar neoplasms (Studies II and IV) and mobile tongue cancer (Study IV), the Swedish Cancer Registry was used. The patients were identified in the database by using the ICD-7 codes for tonsillar cancer (145.0) and tongue cancer (141.7, 141.8, 141.9). The registry was established in 1958 and covers the whole Swedish population; 50 000 cases of different cancer forms are registered each year. Health care providers in Sweden are required to report to the registry any detected new cancer cases diagnosed at clinical-, morphological-, and laboratory examinations, including cases identified during autopsy. Currently, there are six regional registries in Sweden which update the National Cancer Registry with data regarding registration, coding and major check-up of newly detected cases. Three different types of information are included in the Swedish Cancer Registry. 1) Patient data (personal identification number, sex, age and place of residence). 2) Medical records (site of tumor, histological type, stage, basis of diagnosis, date of diagnosis, reporting hospital or department, identification number for each specimen). 3) Follow-up data (date and cause of death or date of migration).

We received approval from the BioBank North to access the accurate samples for the study (472-13-08 in 2013-03-26). Biobank North is administrated by the Laboratory Medicine in Västerbotten and it is responsible for all research sample collections and storage within the unit and that the material is available for each specialty in laboratory medicine. The BioBank North also promotes new collection and increase accessibility to existing tests.

3.8. Statistical analysis

All analyses were performed using SPSS (Statistical Pack for Social Sciences, Inc., Chicago, IL, USA). Descriptive statistics were provided as means with standard deviation (SD). Non-parametric two-tailed tests were used and $p<0,05$ was considered to indicate a statistically significant difference.

3.8.1 Study I

The Mann-Whitney u-test was used in Study I when correlating the patient ages between different subgroups to VHI scores, frequency of treatment sessions, gender, HPV subtype and onset of the disease. In order to assess how well the relationship between VHI questionnaire and the subscales of SF-36 could be described, we used the non-parametric measure of statistical dependence between two variables, the Spearman's rho correlation coefficient. The non-parametric Kolmogorov-Smirnov test was used to compare the VHI results with a reference (published normative values) probability distribution. The Wilcoxon signed-rank test was applied to compare the results of the SF-36 survey, since the normality assumption was not verified.

3.8.2 Study II

The population of northern Sweden as of 2000 was used for age standardization of tonsillar cancer over the period 1990-2013. To compare patient ages between different genders and HPV status (positive and negative), the Mann-Whitney u-test was performed. The Chi-square test was applied to determine the differences between HPV-status and gender.

3.8.3 Study III

The non-parametric Kolmogorov-Smirnov test was used to assure the normal distribution. An expected normal distribution justified the use of the Student's t-test; if not a normal distribution was likely we used the non-parametric Wilcoxon signed-rank test. The limited subpopulation sizes at specific comparisons are urging caution in the statistical interpretation.

3.8.4 Study IV

For the calculation of p values, Chi^2-test was used. For survival analysis, 2- and 5-year survival was used.

3.9 Ethical considerations

The study design was approved by the Regional Ethical Review Board of Umeå University (2012 276 32M, 2010 277 31, 08-003M and 03-201). To access the accurate retrospective paraffin-embedded samples from the BioBank North, we used data from the Swedish Cancer Registry database. The application was approved by the Biobank North (472-13-08 in 2013-03-26). In the prospective Studies I and III, written information was provided to all study participants. The studies were conducted in accordance with the Declaration of Helsinki (2014). (232) The ethical assurance for the retrospective Studies II and IV lies in approval from both the Regional Ethical Review Board of Umeå and the BioBank North.

4. Results

4.1. Study I

A total of 27 patients (82% response rate) completed the questionnaires. No significant differences were observed when comparing the patients included in the study with those excluded. Fifteen patients with RRP (56%) received less than one treatment session per year (low-frequency group – LF), while 12 patients (44%) had more than one or more operations per year (high-frequency group – HF).

In terms of voice quality and certain aspects of quality of life, the RRP group highly deviates from vocally healthy controls. Results are summarized in Table 7.

Table 7. Summary of findings in voice and quality of life aspects in RRP patients.

	VHI$_{total}$	**SF 36 survey** *Social functioning*	**VHI survey** *Voice dysfunction**	**VHI survey** *Minimal voice handicap*	**VHI survey** *Moderate or severe voice handicap*
RRP	p<0,001 vs. normative value	p=0,029 vs. normative value	78%; n=21	41%; n=11	59%; n=16

*According to the normative value of Sw-VHI (voice dysfunction when VHI$_{total}$ >20 points).

Patients that underwent more than one operation per year were younger (41 years vs. 51 years, *p*=0,028) than those treated less frequently. Females, patients with frequent surgical treatments and patients with the high-risk HPV types scored significantly lower in several domains of the quality of life assessment compared with normal subjects. The results are interpreted with caution due to the limited number of subjects. Two patients were positive for HPV16 genotype, both with histopathological squamous dysplasia, and one developed a malignancy with a T1bN0M0 classification.

4.2. Study II

There were 214 cases of tonsillar cancer identified, 155 (72,4%) men and 59 (27,6%) women. The total incidence of tonsillar cancer in northern Sweden (age-standardized to the population of northern Sweden as of 2000) doubled from 0,69/100 000 (year 1990) to 1,38/100 000 (year 2013). More specifically, a 2,7-fold increase (0,83 to 2,25 per 100 000) was noticed in men, while the female group showed only a small rise (from 0,46 to 0,48 per 100 000).

65 biopsy specimens obtained between 2000-2012 (median age 58 years, mean 59,3 years, range 45-87) were analysed; 48 (74%) belonged to males (median age 57,5 years, mean 57,6 years, range 45-74) and 17 (26%) were females (median age 65 years, mean 64,1 years, range 45-87). Female subjects were significantly older (p=0,016). 91% of the specimens (59/65) were positive for HPV DNA and 62 (95%) expressed p16. Of the p16-positive samples, 7 (11%) received the highest score of 18 points, 49 (79%) a score of 12 (intermediate intensity) and 6 (10%) samples received between 2-6 points.

4.3. Study III

Twenty-seven of the 48 eligible patients in Study III were also analysed in Study I. The median duration of the disease was 7,2 years (men 8 years, females 6,5 years). A detailed outline of the resulting demographics is provided in Table 8, sample characteristics are presented in Table 9, prominent data are highlighted in bold lines. RRP patients with high surgical treatment frequency were significantly younger and had a more widespread laryngeal disease compared to a low-frequency treated group.

Table 8. Outline of resulting demographics of RRP in Study III.

Demographics	**n (%)**
Total number, n=48 (median age 44,5 years)	
Females (median age 48 years)	14 (29%)
Males (median age 43,5 years)	**34 (71%)**
Juvenile-onset	6 (12%)
Adult-onset	**42 (88%)**
HPV6	**32 (67%)**
HPV11	5 (10%)
HPV16*	2 (4%)
HPV31	1 (2%)
Not determined HPV subtype	8 (17%)
Glottic papilloma	**48 (100%)**
Supraglottic papilloma	7 (15%)
Subglottic papilloma	5 (10%)
Vocal fold web	5 (10%)
Cancer development	1 (2%)

*One out of two patients developed malignancy, receiving radiotherapy.

Table 9. Sample characteristics of RRP in study III.

	High-frequency (HF)	Low-frequency (LF)	p-value (t-test)
Age	**Median 37 years**	Median 46 years	0,04
Number of surgeries/year	**Median 1,6**	Median 0,4	0,02
Glottic papilloma (n=48)	17	**31**	NS
Supraglottic papilloma (n=7)	**5**	2	0,03
Subglottic papilloma (n=5)	**5**	0	0,02
Juvenile-onset (n=6)	3	3	NS
Adult-onset (n=42)	13	**29**	0,002
Web (n=5)	3	2	NS

NS=non significant.

Adult-onset of the disease was more common in the LF group (p=0,002). Without scientific support for the efficacy of therapeutic vaccination, eight patients chose to be vaccinated, and seven of them belonged to the HF group. We observed a trend towards reduced necessity of surgery in the vaccinated subgroup of patients. Figure 9 reflects this conclusion by demonstrating the benefit of the 3-step vaccination process (Gardasil®) in one single individual patient.

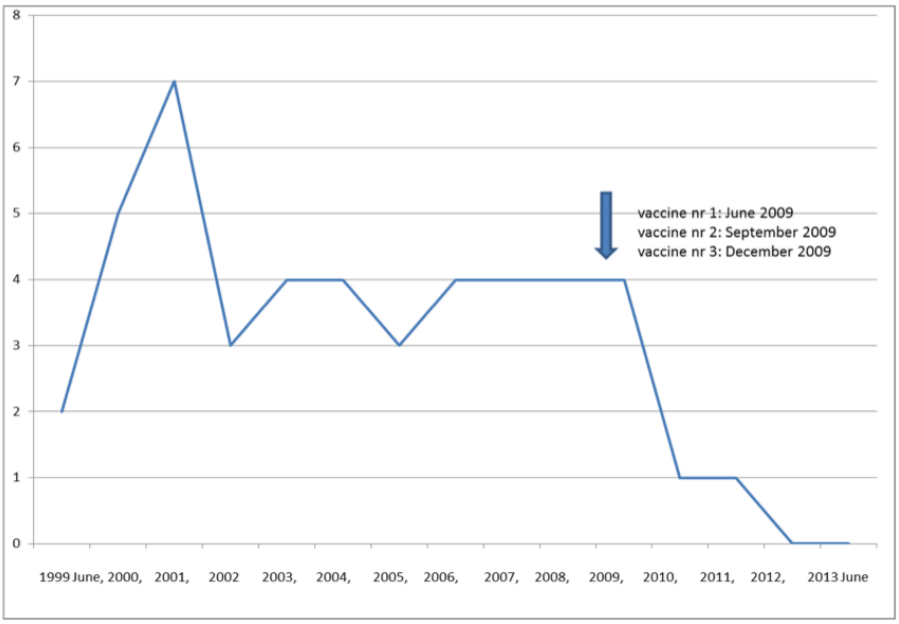

Figure 9. Outcome of 3-step HPV vaccination process in one individual, Study III. The Y-axis indicates the number of treatment sessions per year, and the X-axis the timeframe longitudinally.

4.4. Study IV

109 patients with TSCC were eligible for participation in the study; 54 were men and 55 were women (mean age 63,5 years, range 19-93 years). The sample characteristics are presented in Table 10. Two-thirds of the samples were obtained from the lateral border of the mobile tongue, 20% from the ventral side and 2% from the dorsal side. Lower survival and disease-free rate was noticed in patients in the young age group (≤40 years) compared with the older (<65 years) patients.

Table 10. Sample characteristics in mobile tongue cancer, Study IV.

Age at diagnosis (years)	Number	Male/female ratio	T1	T2	T3	T4	N0	N+	M0	M1
≤40	16 (14,7%)	7/9 1:1,3	2	10	2	2	12	4	16	0
41-65	38 (34,9%)	26/12 2,2:1	14	11	9	4	27	11	37	1
>65	55 (50,4%)	21/34 1:1,6	15	19	8	13	41	14	54	1
	109		31	40	19	19	80	29	107	2

M: distant metastasis; N: nodal metastasis; T: tumor size.

67% of the tumor samples were negative for the expression of p16 marker, independent of site of lesion. HPV was not detected in any patients. All samples analysed for syndecan-1 expression were positive while there was no correlation between p16, syndecan-1 expression or HPV in mobile tongue cancer.

5. Discussion

5.1 Specific aims and findings

This thesis aims to increase our understanding of HPV in recurrent respiratory papillomatosis (RRP), tonsillar cancer, and mobile tongue cancer. These are diseases with care-intensive treatments, sometimes life-long treatment courses, and in most cases, major functional complications affecting voice, speech, swallowing and quality of life. We want to provide a scientific basis for an introduction of gender-neutral vaccination, by studying patients with recurrent respiratory papillomatosis and the longitudinal increasing development of HPV-related tonsillar cancer in northern Sweden.

5.1.1 Impact of HPV in malignant and benign lesions

RRP and tonsillar cancer are HPV-related in contrast to cancer in the mobile (oral) tongue. The HPV-related airway disease affects mainly young/mature men and involves mainly the genotypes HPV6 and HPV16. There is a correlation between HPV and its surrogate marker p16 in tonsillar cancer but not in the HPV-negative mobile tongue cancer cases.

In Study II, we noted a gender-neutral 2,7-fold increase in the incidence of HPV-related tonsillar cancer cases in males between 1990-2013. This is in accordance with results of previous studies that suggested a global increase of the incidence of HPV-positive tonsillar cancer, preferentially in males. (153, 233-235)

Recent changes in sexual patterns, such as increasing numbers of sex partners, practice of oral (mainly) or vaginal sex, and early debut of sexual activity have been proposed as possible explanations for the increase in HPV-positive tonsillar cancer. (236) The increase of HPV-related tonsillar cancer cases in men is unclear, but we propose that there is a possible gender difference in sexual-, alcohol- and smoking behaviour. The hypothesis has found scientific support in previous studies reporting a connection between reduced clearance of oral HPV in men associated to heavier tobacco and alcohol use. (237) Females seem to have a stronger immune response due to exposure to genital HPV earlier in life. (237) Furthermore, HPV has been suggested to be more easily transmitted from women to men than the opposite way during sex, though why this might be is unknown. (238)

The majority (95%) of the tonsillar tumor specimens indicated an overexpression of the protein marker p16, consistent with previous findings. (162, 239) Thus, p16 can be used as a surrogate marker and a prognostic guide in specific HPV-related malignancies. The lack of correlation between p16 and HPV in mobile tongue cancer shown in Study IV indicates that HPV-induced squamous cell cancer is a

site-specific disorder in the upper aerodigestive tract. This is in contrast to Begum and Gillison (2003) suggesting that p16 could be used as a marker to determine whether cervical lymph node metastasis originates from the oropharynx, since they found a credible correlation between p16 and HPV. (240) The HPV-related tonsillar malignancies have shown a better prognosis regarding overall survival and locoregional control compared to HPV-negative cases. (241, 242) De-escalation of the aggressive treatment offered to HPV-positive tonsillar cancer cases has therefore been proposed, in order to preserve organ functionality. (243, 244) It is important to note that improved survival rates of HPV-infected tonsillar cancer cases have not been observed in patients diagnosed with HPV malignancies originating from other sites of the head and neck. (245)

In Study III, a cohort of RRP patients recruited from the northern Sweden catchment area was studied prospectively. The predominance of males in the RRP disease is consistent with the gender profile of tonsillar cancer, a fact that needs to be addressed in light of the current vaccination strategy The division of patients into low-frequency (<1 treatment sessions/year) and high-frequency groups (≥1 treatment sessions/year) was applied to help elucidate an aggressive disease profile characterized by more widespread airway lesions and a higher frequency of treatment sessions. In contrast to some previous reports, no subpopulation was identified with an aggressive course related to juvenile-onset and HPV11. (100) This conflict in outcome could be explained by the small numbers of study patients and thus, reduced reliability. HPV colonisation in the oral mucosa is much higher in AoRRP than JoRRP with a prevalence of less than 7%. (193) This could also indicate different transmission routes depending on age. (51)

As previously reported, HPV-positive tonsillar carcinomas are currently considered to be clinically and biologically different tumor entities as compared to HPV-negative cases. (160) The role of HPV in development of laryngeal cancer has not been definitely established. However, referring to a population-based cancer registry study, HPV may be involved in the development of a subset of laryngeal cancers, especially in females. (60) The HPV16 genotype is undoubtedly considered a high-risk virus linked to cancer development in the airway, and has been identified in 4% of our RRP patients. Today, the existence of HPV infection in laryngeal cancer does not affect the current treatment regime.

Treatment of RRP currently relies on surgery and aims for symptom reduction since curative interventions are still lacking. In light of the fact that we are not able to offer curative treatment options for RRP, we have identified clinical guidelines to focus on particularly care of specific high-risk RRP patients (Study III) and moreover, to understand the impact of the disease regarding patients' quality of life, social network and affected ability to work full time in voice-profiled professions

(Study I).

In contrast to Studies I, II and III, HPV could not be detected in mobile tongue cancer (TSCC) (Study IV), despite the use of the highly sensitive in situ hybridization. This finding could either indicate that HPV is absent in TSCC, or present at extremely low levels; despite this, it could be possible that other high-risk subtypes of the HPV virus could be present. We do not believe that cancer of mobile tongue is HPV-induced, supported by low numbers of HPV infection in normal oral mucosa (246) and the outcomes of previous studies. (247, 248) It seems unlikely that HPV constitutes a significant factor in the pathogenesis and rising trends of TSCC in the young population in contrast to the geographically adjacent tonsillar cancer cases; the HPV virus simply prefer tonsils.

5.1.2 VHI and SF 36

RRP patients have been reported to have inferior health-related quality of life and voice deterioration compared to non-RRP matched data. (249) VHI has been considered as an appropriate instrument to validate vocal impairment. It is designed to measure the effects of social and psychological damage due to voice disorders. (222, 250) SF-36 was employed since it is a widely accepted instrument to assess daily activities and measure several aspects of the quality of life. (251)

In Study I, age and the predominance of men were strongly correlated with the frequency of treatment results in accordance with other RRP studies. (99) 11% of patients in Study I exhibited the high-risk subtypes 16 and 31. These patients reported a significant deterioration regarding several aspects of health-related life quality compared to the majority of the patients with the low-risk subtypes 6 and 11. One of the patients with HPV16 converted to malignancy, highly affecting the quality of life and voice standards in the small subgroup analysed. Another gender disparity was that females reported lower scores in different subscales of the SF-36 questionnaire, revealing an overall deterioration of life quality. These outcomes have to be analysed in the light of low numbers of females of which one developed a malignancy, highly affecting the quality of life. However our results are not contradicted by previous reports. (252)

The correlation between voice disorders and quality of life, and more specifically social functioning, is reflected in Study I where significantly lower values were obtained in RRP patients as compared to the normal population. Similar findings were established by other researchers. (249, 253) The impact of voice regarding the quality of life is more extendedly studied in AoRRP compared to JoRRP; the latter was characterized by a quality of life similar to that of children with other chronic diseases. (254) These findings are in line with those in Study I, where significant

impairment of voice quality was shown among small number of juvenile-onset RRP patients.

The majority of RRP patients experienced voice deterioration. Moderate to severe dysfunction was common in the RRP patients, while 22% reported normal voice quality. The perception of normal voice quality is complex, but may be explained by a low vocal burden of the disease affecting the vocal cords, patient's expectation effect, low active RRP disease profile, voice quality in relation to voice load in daily life, and of course the effect of small-sized population analysed, undermining the reliability. The heterogeneous voice outcome in RRP patients is supported by Ilmarinen et al. (255) The scientific diversity is consistent when studying voice outcome and quality of life in the RRP patients. We were unable to detect any association between the frequency of surgical treatments and voice quality. This was in agreement with Lehto et al., reporting that age but not number of procedures correlated with poor voice quality among patients. (256) However, Ilmarinen et al., reported a clear-cut connection between high number of laryngeal procedures and deterioration of voice quality, (255) Lehto et al. concluded that voice quality in RRP patients is not significantly worse than it is in healthy control subjects, even if parameters like roughness and breathiness were observed in the perceptual assessment. (256) This is a reasonable conclusion since the RRP disease and the voice quality over time will be integrated as a normal state of being and sounding for the individual and its social context. This diversity highlights the need for studies with larger sample sizes and higher statistic power to detect consisting reproducible results.

5.1.3 Importance of p16 and syndecan-1

In Study II, we observed an overexpression of protein marker p16 in the tonsillar cancer specimens, demonstrating a strong association between p16 and HPV infection in tonsillar carcinomas and the use of p16 as a prognostic guide. In Study IV, p16 was expressed in some cases of mobile tongue cancer (TSCC), however HPV was undetectable. Therefore, p16 should not be used as a reliable surrogate marker for high-risk HPV infection in mobile tongue cancer, despite the presence of the HPV-receptor syndecan-1. HPV simply prefers the tonsillar environment. Lack of p16 is associated with worse prognosis primarily in young patients with TSCC. (257) Molecular alterations in the p16 pathway, independent of HPV infection, could explain the p16 overexpression. Alternatively, the presence p16 in HNSCC could be the truly important prognostic marker, independent of HPV status, and could alone constitute an important prognostic marker in HNSCC. Some researchers support the argument of using p16 than direct HPV-testing in tonsillar carcinomas. (258)

Expression of the HPV receptor syndecan-1 did not exhibit any difference between the TSCC group and tonsillar cancer (Studies II and IV). The initial binding of HPV virus to the cellular surface of both tongue and tonsillar tissues could theoretically take place in a similar way. However, as 91% of the tonsillar cancer cases were HPV-positive, we could conclude that the virus presents with a preference for tonsillar environment. We assume that this tonsillar predilection of the virus could enlighten the value of co-infection with other viruses such as HSV and EBV. (259, 260) There are conflicting studies concerning the expression of syndecan-1´s role in a tumor recurrence and tumor-specific death in oral carcinomas. (261, 262)

5.1.4 HPV and vaccines

The clinical utility of HPV vaccination as a prophylactic measure for cervical cancer in women is well established. Our hope is that through the on-going vaccination strategy towards cervix cancer we will end up with a reduced incidence of tonsillar cancer and RRP. (89) The incidence rates of tonsillar cancer are rising worldwide. We can probably expect a further increase of HPV-related tonsillar cancer in parallel with a further decline of cervical cancer due to preventive HPV vaccination in females. (263)

HPV vaccines constitute a great opportunity to potentially prevent tonsillar cancer in future generations. In light of the growing incidence of male HPV-related tonsillar cancer and the predominance of males in RRP, the argument for using HPV vaccines in boys becomes more forceful. A study group has conducted a 'Safety Study of HPV DNA Vaccine to Treat Head and Neck Cancer Patients'. The study was completed in April 2015 but no results have been published to date. (264) Previous studies have reported that men will benefit directly from vaccination of girls, but men remain at an unfortunately high risk of developing HPV-related cancer. (89) The efficacy of HPV vaccine against oral HPV infection has been studied by Herrero et al., reporting a 93,3% reduction in the rate of oral HPV16/18 infection. (265)

The outcome of the vaccine-preventable RRP burden has not been thoroughly studied. Freed and Derkay (2006) stated the expected positive outcome on reduced incidence of RRP in relation to vaccination with Gardasil® vaccine. (137) A first attempt to take advantage of the preventive role of HPV vaccination against RRP on a case level was published by Förster et al. using Gardasil® in order to reduce the aggressive course of RRP in a 2-year old boy. Successfully, the disease became stable after the third immunization and no surgical treatment was necessary for the following ten-month period. (266) Hocevar-Boltežar et al. studied the efficacy of Gardasil® vaccine in a population of 11 patients (aged 13-46 years) with an aggressive RRP course. The results showed extended treatment intervals and consequently, a reduction of necessary surgical sessions. (138) One of the patients in

that study showed complete remission of the disease after vaccination. The effectiveness of vaccination with increased treatment intervals and lower treatment needs has been reported in small-scale studies. (91, 133, 139)

In Studies I and III, we identified a more care-intensive subgroup of RRP patients. In light of the on-going therapeutic vaccination evaluation, we advocate an initial selection of patients for HPV vaccination based on male gender, younger age, more widespread airway disease, and oncogenic HPV genotypes (Studies I and III). Eight of our RRP patients in Study III chose to receive HPV vaccine, and seven of them belonged to the high-frequency group. We observed a trend towards less surgical treatment in this small cohort, results consistent with previous reports. (91, 133, 137-139, 266)

In Study II, we reported a 2,7-fold incidence increase of tonsillar cancer in male patients. In northern Sweden, a male incidence polarization was also obvious in our RRP material (Study III). Therefore, it could be beneficial to evaluate the efficacy of HPV vaccination as a prophylactic measure against both tonsillar cancer and RRP in the male population.

Finally, we briefly want to address awareness of the difficulties with small size studies. A study with low statistical power has an obvious reduced chance of detecting a true effect. The consequence of this is a tendency to overestimate effect size and a low reproducibility of results. There are also ethical dimensions of the problems with small population studies such as unreliable reproducibility as a major methodological principle.

5.2 Clinical implications

RRP patients experience a significant voice dysfunction, affecting quality of life as well as social habits and working capacity. Measuring the influence of RRP on voice and quality of life offers the possibility to identify valuable prognostic factors of the RRP disease. The frequency of RRP surgical interventions, age at onset, gender, and subtype of the HPV may be used as factors to predict voice disability. Patients with the high-risk HPV genotypes are especially vulnerable. The high-risk HPV genotypes do not seem to affect their treatment frequency, but their perception of reduced quality of life and voice.

We have identified a more care-intensive RRP subgroup defined by frequent need of treatment sessions, younger age, male gender and more widespread disease in the airway. This subgroup is not primarily associated with JoRRP or HPV11. The study outcomes can be used in clinical practice in order to inform the RRP patients about the disease upon diagnosis, predict the course of RRP based on clinical characteristics, and become a basis for vaccination discussion.

The increasing trend in the incidence of tonsillar cancer in northern Sweden by 2,7 fold in men, as well a worldwide increase of increase of HPV-positive tonsillar cancer cases, highlights the need of clinically applicable simple tests to detect HPV-related malignant tonsillar disease. There is a strong association between p16 and HPV infection in tonsillar cancer. The overexpression of p16 is highly suggestive of HPV infection in tonsillar cancer, and can be used in clinical practice as a surrogate marker and a prognostic guide of tonsillar cancer but not in mobile tongue cancer. HPV was undetectable in mobile tongue cancer while p16 is expressed in a few cases. Therefore, p16 is not a reliable surrogate marker for HPV infection in mobile tongue cancer. It may serve alone as a prognostic marker, since the lack of p16 is associated with worse prognosis.

This thesis has contributed scientifically with simple tools to detect HPV in tonsillar cancer. It has also presented findings supporting a gender-neutral vaccination in defined RRP subgroups and in tonsillar cancer, but not in mobile tongue cancer.

5.3 Limitations

This thesis was limited by several factors. Firstly, although all patients with RRP in the catch-up area were eligible, the general number of patients was low. This was even more noticeable when statistically analysing the high-risk HPV subtypes, supra- and subglottic HPV distribution and juvenile-onset. There were not enough observations to support confident conclusions. Secondly, an important limitation, but also an asset which applies to Studies I, II and III, is the fact that our patient material was restricted geographically in the area of northern Sweden.

Thirdly, our patient material in Study I was compared to the general, historical Swedish normative values for VHI and SF-36, where no data on the variation of the normative values was available over time. Additionally, the patients' vocal ability was not evaluated pre-operatively, for comparison purposes. A multimodal assessment would have been desirable. Due to the care-intensive life conditions, and in order to reduce the possibility of discontinuation to participate, we wanted to minimize the documentation as much as possible. This was balanced with the need to preserve validity in terms of the relevant level of detail for the registrations performed.

Finally, there was a lack of a comparable cohort including patients from different regions and assessing subject characteristics at specific time points of their disease. This can be considered an additional limitation in all studies, except Study IV where patients from another country (Italy) were recruited.

5.4 Future perspectives

In order to increase the statistical power, the true effect, the reproducibility of the results and the conclusions of this thesis, further long-term multicentre studies with larger intervention groups are required. In light of the reported efficacy of the HPV vaccine, we could study the effects of vaccination in selected RRP patients longitudinally and in a multicentre design, with effects on surgical intervals and local aggressiveness of RRP lesions. A further purpose with an expanded study design is to investigate the routes of HPV transmission in the airway and the mechanism of mucosal RRP lesion penetration in the larynx of RRP patients.

Additionally, it would be interesting to study patient cohorts with nasal inverted papillomas, given the lack of knowledge around the cause of the disease. It is relevant to understand why, despite its benign histopathology, this disease entity has a malignant clinical course. If we could establish that HPV is present in inverted nasal papilloma then this could have impact on vaccination and affect the protective environment in the operation theatre. Moreover, it would be interesting to study the presence of HPV and other possible viral co-infecting agents in non-malignant tonsils, since we are facing a major increase in HPV-positive tonsillar cancer. This would potentially help to understand the relation between the “time of infection” and the “onset of malignant tonsillar disease”. Finally, high-quality, randomized control trials could be initiated in order to address the most effective treatment methods for both HPV-positive and HPV-negative tonsillar cancer patients.

6. Conclusions

- The frequency of operations, age at onset, gender and genotype of human papillomavirus (HPV) may be used as factors to predict voice disability in patients with recurrent respiratory papillomatosis (RRP). The majority of the patients with RRP experience a significant self-reported voice dysfunction and in specific domains, a clear-cut significant impact on quality of life. A subgroup of RRP patients represented by low age, females, high-risk HPV genotype and high frequency of treatment sessions seems to be more vulnerable for morbidity in terms of quality of voice and life.

- There is a parallel increase in the incidence of tonsillar cancer, HPV infections and expression of p16 among patients from northern Sweden, confirming the strong association between p16, HPV infection and tonsillar cancer.

- RRP patients with high surgical treatment frequency are younger men, infected with HPV6 and have a widespread laryngeal disease. This indicates a clinical subgroup of RRP patients, not primarily related to HPV subtype, but to a more care-intensive course. The majority of RRP patients in northern Sweden are men, with an adult onset and infected with HPV6.

- HPV is undetectable in mobile tongue cancer; p16 is expressed occasionally, indicating poor correlation between HPV and p16 in tongue cancer. p16 is not a reliable surrogate marker for HPV infection in mobile tongue cancer but may serve alone as a prognostic marker since the lack of p16 is associated with worse prognosis in younger patients (≤40 years).

7. Acknowledgments

The completion of my PhD thesis has been a long journey. I would like to express my sincere gratitude to everyone that has helped, encouraged and supported me on my scientific journey during the last four years.

In particular, I would like to thank:

Katarina Olofsson, my main supervisor, for your gentle and positive academic and scientific guidance. Many thanks for your friendship, support and encouragement in times of doubt, for your great experience in research and for teaching me what research is all about: to understand how things really work. You have been a steady influence throughout my thesis; you have oriented and supported me with promptness and care. Thank you for believing in me and your help on generating and managing the projects of this thesis. Without you, nothing of all these could be accomplished.

My co-supervisors, Karin Nylander, David Lindquist and Göran Laurell. Thank you for your scientific skills and your wide knowledge, your patience during our constructive and productive meetings and discussions. Big thanks for helping me solving problems every time a problem showed up.

Charlotte Öfverman and Kristina Stefansson, for all your excellent work and assistance. Thank you for all your help with discussing different lab methods and other interesting issues. Your help in putting numbers into words has been invaluable, many thanks!

Andreas Arvidsson, dear friend and colleague. Thanks for your help, knowledge and wise comments, despite a high work load in the clinic.

Anna Holm, colleague and co-author. Many thanks for being a patient co-researcher and for boosting the project with your positive energy and everlasting enthusiasm. Good luck with your thesis!

Professor Michael Haney. Thank you for the critical analysis and comments on the linguistic aspects of the thesis.

Anders Lundquist, for your important statistical advices and encouragement.

My gratitude is also extended to all the colleagues and friends at the ENT department, University Hospital of Umeå and especially, my ‘roommate’

Mohammad. Thank you for a great working atmosphere, stimulating help and professional attitude. You made work such a nice place for me.

Our fantastic patients, for their patience and willingness to participate in these studies.

Costas and Marios, my fellow countrymen and great friends in Umeå. Big thanks for your friendship, support and endless optimism and for cheering me up with your company and jokes all these years.

My parents for their support and everlasting love; I am eternally grateful for everything you have done for me all these years! You have instilled many admirable qualities in me and given me a good foundation with which to meet life. You've taught me about hard work and self-respect, about persistence and about how to be independent.

My siblings, Marilena and Louis. Thank you for your infinite support throughout everything.

Many thanks to all the other members of my big, fantastic family.

My two little angels, Elin and Alexander. Thank you for your encouragement through looking at your beautiful smiles. You're all the bright stars in my sky, the music in my life, the air I breathe!

Last but not least, my wife and partner in life, Linda. Thank you for your energy, love and support. For sacrificing your time in taking care of our children all those days and nights I was away preparing this PhD thesis. Through your patience and unwavering belief in me, I've been able to complete this long dissertation journey. You are my biggest fan and supporter. I am endlessly blessed for you being in my life!

This thesis was supported by grants from Lion's Cancer Research Foundation at Umeå University, CFF, LP12-1945, CFF LP13-1998, CFF, LP14-2048 and CFF AMP 15-772 and Umeå University Medical Faculty, Sweden.

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Acta Oto-Laryngologica. 2014; 134: 401–406

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ORIGINAL ARTICLE

Voice and quality of life in patients with recurrent respiratory papillomatosis in a northern Sweden cohort

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Abstract

Conclusion: The frequency of operations, age at onset, gender and subtype of the human papilloma virus (HPV) may be used as factors to predict voice disability. *Objectives:* Patients with recurrent respiratory papillomatosis (RRP) are characterized by morbid consequences due to a lifelong repetitive influence on voice and breathing ability and the need for recurrent surgical treatments. The aim of the study was to measure the quality of voice and life using evaluated and validated questionnaires in a northern Sweden RRP population. *Methods:* A total of 27 consecutive patients with RRP (age 21–71 years, median 47 years) were evaluated 3 months postoperatively (CO_2 laser treatment) using the voice handicap index (VHI) and SF-36 questionnaires to assess the impact on life and voice in an RRP population. The values were compared to historical normative data, VHI ≤ 20. *Results:* Patients that underwent more than one operation per year were younger ($p = 0.028$) than those treated less frequently. The mean VHI_{total} score in patients with RRP was 39.3, indicating a statistically significant impairment of voice quality ($p < 0.001$) as compared with normal subjects. Voice dysfunction was observed in 21 patients (78%). Significantly lower values than the normal population regarding the quality of life in patients with RRP were obtained in the domain of social functioning ($p = 0.029$). Females, patients with frequent surgical treatment sessions and patients with the high-risk HPV types scored significantly lower in several domains of the quality of life assessment as compared with normal subjects. The results should be interpreted with caution due to the limited number of subjects.

Keywords: *Human papilloma virus, RRP, VHI, SF-36*

Introduction

Recurrent respiratory papillomatosis (RRP) is a rare disease of viral aetiology that occurs in both children and adults. Cases are grouped into juvenile and adult-onset forms. The incidence in the United States is estimated to be 4.3 per 100 000 children and 1.8 per 100 000 adults [1], which if given the same population prevalence in the northern part of Sweden (approximately 850 000 inhabitants) would mean that new cases of RRP would be expected in 49 adults and 20 juveniles each year. The most common human papilloma virus (HPV) subtypes in RRP are HPV6 and HPV11 [1]. Although categorized as a non-malignant disease, RRP has a high morbidity impact due to symptoms affecting the voice and, in severe cases, breathing capacity [1]. The overall estimated number of different HPV species is nearly 200 [2].

The disease has a significant recurrence rate, also sometimes with high-risk HPV subtypes (16, 18, and 31) that are recognized to be an important factor for malignant cell transformation [3,4]. Currently there is no medical or surgical cure available for RRP. The natural history and clinical course of the disease can differ widely between patients. In some subjects, a long-term remission can be quickly achieved after

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(Received 8 October 2013; accepted 13 November 2013)

ISSN 0001-6489 print/ISSN 1651-2251 online
DOI: 10.3109/00016489.2013.867457

surgery, while others need repetitive treatments with short intervals in between. The reason for this variation in clinical course is not known.

The current therapeutic strategies are based on removal of the wart-like bulky papilloma lesions using a carbon dioxide (CO_2) laser [5], microdebridement [6] and radiofrequency coblation [7]. Adjuvant therapies, including α-interferon, indole-3-carbinol and cidofovir, have been used with low success rates and frequent unwanted side effects [8–10]. The use of vaccines and immunization strategies may be promising in the treatment of RRP in the future [11], although the quadrivalent Gardasil® has not yet been proven effective in established HPV infection caused by HPV (types 6, 11, 16 and 18) and is currently only indicated for prophylaxis [12].

Our primary goal was to measure the effects of RRP on voice and quality of life for patients treated at a single regional referral centre for northern Sweden and to compare these findings to Swedish national values, representing the general Swedish population. We aimed to measure the impact of RRP on lifestyle and to assess the relation of early manifestations of the disease to long-term morbidity.

Material and methods

Patients

The study was approved by the Regional Ethical Review Board in Umeå (2012-276-32m, 2010-277-31m). This was a prospective questionnaire-based cohort study where 27 consecutive, non-smoking patients with RRP (median age 47 years, range 21–71 years) were enrolled based on a diagnosis of RRP and surgical treatment with CO_2 laser technique between 2004 and 2012 at the Department of Otorhinolaryngology, University Hospital of Umeå, Sweden.

Study design

Subjects were selected and offered enrolment only if they had not been previously tracheotomized, vaccinated with HPV vaccines or treated with optional therapies such as α-interferon, indole-3-carbinol or cidofovir. Further exclusion criteria included previous surgical treatment of RRP with microdebridment or radiofrequency coblation during the last 10 years. Subjects were enrolled after receiving information on the details of the study and then providing consent. Postoperatively they were assessed during an outpatient visit within 8 weeks after surgery.

The patients received the questionnaires, the Voice Handicap Index (VHI) [13] and the Rand Short Form (36 question) Health Survey (SF-36) [14], within 3 months after the last treatment session. If the questionnaires were not returned, one reminder was dispatched after 6 months.

Evaluation and recordings

Preoperative videostroboscopy was performed with Olympus ENF P4 or rigid endoscopic 70° model 8705CJ (Storz Hopkins, Tuttligen, Germany) with stroboscopic function. The evaluation included visualization of the larynx, vibration capacity of the vocal folds and the papilloma distribution. The location and distribution of papilloma vegetation in the larynx were categorized into supraglottic, glottic, subglottic and tracheal areas.

Intraoperatively, biopsy was obtained for histopathological studies and HPV identification. A papilloma tissue sample was kept in sterile saline; the tube was sent to the Clinical Microbiology Laboratory at Skane University Hospital in Malmö for analysis. Polymerase chain reaction (PCR) products were analyzed for specific genotypes using the Luminex system that contains type-specific probes for mucosal HPV types (high-risk, e.g. 16, 18, 31, 33 and low-risk, e.g. 6 and 11). The laboratory is accredited according to the ISO 15189 standard for 14 oncogenic types and for HPV6 and HPV11 used in the HPV test. The Luminex assay also included two broadly reactive 'universal' probes and samples positive only for a universal probe were typed by sequencing. The standard operative treatment for all patients was ablation/vaporization using CO_2 laser [15].

For individual patients, the decision to proceed to operation was based on subjective voice discomfort, perceptual voice deterioration and the videolaryngostroboscopic findings.

Voice handicap index

The effect of voice disorder on daily life was assessed using the VHI questionnaire to serially measure the patient's perception of voice quality, before and after treatment for laryngeal disorders. In this survey [13], 30 items are examined and grouped into three subscales relating to voice disorders: physical, emotional and functional aspects. Each of the 30 questions will elicit a response and corresponding score: 0 for 'never', 1 for 'almost never', 2 for 'sometimes', 3 for 'almost always' and 4 for 'always'. Each subscale comprises 10 items. A total VHI score ranging from 0 to 120 points is obtained by summarizing the scores for each category. A voice handicap score from 0 to 30 represents 'minimal' handicap, from 31 to 60 'moderate' handicap and 61 to 120 points indicates 'serious' voice handicap. A validated Swedish translation of the VHI

questionnaire [16] has been reported (with a threshold score of 20 as an approximate normal score in the Swedish population).

Quality of life

The SF-36 questionnaire was employed for the assessment of quality of life [14]. This instrument examines eight different domains: physical functioning, role limitations due to physical health and emotional problems, vitality (energy and fatigue), emotional well-being, social functioning, body pain and general health status. It includes a total of 36 questions that are grouped into these subcategories. Each patient receives a score for each domain, ranging from 0 (worst) to 100 (best). Combining selected domains generates a further two generalized subscales; the physical (PCS) and mental (MCS) component summary scores. The average SF-36 scores from the generalized population in Sweden from 1995 were used for comparison [17]. There is no knowledge about the change over time in the SF-36 measurements.

Analysis

There was no assessment of intra-rater or inter-rater reliability since the diagnostic steps were considered reliable. Statistical analysis was performed using the SPSS (Statistical Pack for Social Sciences, Inc., Chicago, IL, USA) version 17.0. The Mann-Whitney U test was used to compare patient ages between different subgroups, as well as to test for association between the VHI scores (total and subscales), frequency of operations, gender, type of HPV and the onset of the disease. Spearman's rho correlation coefficient was used to test for association between the variables of the VHI questionnaire and the subscales of SF-36.

Finally, after verifying the normality assumption using the Kolmogorov-Smirnov test, a one-sample *t* test was used to test for differences between the VHI and the published normative values. The Wilcoxon signed-rank test was used to compare the results of the SF-36 questionnaire with the published values regarding the Swedish population, since the normality assumption was not met according to the Kolmogorov-Smirnov test. Significance was defined as a p value of < 0.05.

Results

Patients

A total of 27 patients (82%) responded and completed the questionnaires (Tables I and II). The six remaining patients (18%) were excluded from the study since they did not return the questionnaires. No significant differences were observed when comparing the patients included in the study with those who did not complete the two questionnaires in any analysed aspect. Fifteen patients (56%) had less than one operation per year (low-frequency (LF) group), while 12 patients (44%) required one or more treatment sessions per year (high-frequency (HF) group). A statistically significant higher mean age was observed in the LF group (51 years) as compared with the HF (41 years, $p = 0.028$). HPV16-positive RRP patients ($n = 2$) had cancer in situ at the time of diagnosis; one developed a laryngeal squamous cell carcinoma (T1bN0M0) that was successfully treated with curative mono-radiotherapy.

Voice handicap index

In the VHI questionnaire, the mean total score in RRP patients was 39.3 (SD = 24, range 0–85), indicating impairment of voice quality ($p < 0.001$) in the RRP cohort when compared with the normative value for the Swedish population [16]. Six patients (22%) reported normal quality of voice while 21 (78%) experienced voice dysfunction. Eleven patients (41%) experienced minimal voice handicap, eight patients (29.6%) moderate dysfunction and eight patients (29.6%) severe voice handicap. All the patients with juvenile RRP reported a deterioration of their voice; these results have to be observed with caution due to low numbers. There was no difference in VHI_{total} and VHI subscales when comparing the LF with the HF group.

Quality of life

The RRP patients responded with lower values in the domain of social functioning ($p = 0.029$) compared with Swedish normative values. No other differences were detected when comparing the scores from the different domains to the normal population means. The HF treatment group reported a clear deterioration of the quality of life in the domains of physical functioning ($p = 0.015$), emotional role functioning ($p = 0.040$) and the mental component summary score ($p = 0.034$), when compared with patients in the LF group. Females, compared with males, reported lower function in the domains of physical functioning ($p = 0.009$), physical role limitation ($p = 0.014$), body pain ($p = 0.006$), general health ($p = 0.049$) and physical component summary score ($p = 0.008$). Patients infected by the high-risk subtypes (two) reported lower scores in the subscales of social functioning ($p = 0.001$), emotional role limitation ($p = 0.003$) and mental component summary score ($p = 0.003$), compared with RRP patients infected

Table I. Clinical characteristics and demographic data of the 27 study subjects.

Subject no.	Age (years)	Gender	HPV subtype	Disease onset	No. of operations*	Operations/year
1	43	M	6	A	8	1.1
2	44	M	6	A	3	0.4
3	21	M	6	J	14	1.4
4	41	M	6	J	47	2
5	27	M	6	A	6	0.9
6	55	M	11	A	28	1.2
7	60	M	–	A	7	0.2
8	34	M	6	A	7	1.4
9	55	M	6	A	4	0.7
10	47	M	6	A	4	0.3
11	49	F	16	A	8	1.6
12	64	F	11	J	13	0.2
13	47	F	6	A	5	0.4
14	40	F	6	A	2	0.3
15	71	M	6	A	7	0.2
16	26	F	31	J	4	1
17	42	F	–	A	2	0.2
18	63	F	–	A	2	0.3
19	54	M	6	A	3	0.4
20	30	F	6	A	7	1.4
21	34	M	6	A	2	1
22	35	M	–	J	48	1.5
23	55	F	–	J	2	0.5
24	42	M	16	A	2	0.7
25	59	M	6	A	3	0.3
26	61	M	6	A	2	2
27	52	F	6	J	2	2

A, adult onset; F, female; HPV, human papilloma virus; J, juvenile onset; M, male.
*Number of operations performed since diagnosis.

with low-risk HPV subtypes. The current data need to be evaluated carefully due to the low number of patients with high-risk HPV subtypes.

Discussion

These findings from a regional referral centre characterize the effects and influence of RRP on voice quality and quality of life and identify predictor factors and particularly the relationship between voice impairment and impact on the quality of life. Until now, there have been no published reports on the explicit effect of voice quality and impact on quality of life in relation to the frequency of surgery performed, gender and subtype of HPV. The majority of patients (7.8%), particularly juvenile RRP patients, indicated significant voice impairment (VHI_{total} score ≥ 20) affecting their daily activities. However, six adult RRP patients (22%) revealed a normal voice quality (VHI_{total} score < 20). The vast majority of the RRP population in northern Sweden (59%) experienced a moderate to severe voice dysfunction, while the remaining subjects reported minimal voice handicap, consistent with previous studies [18–20].

Our findings showed a strong correlation between treatment frequency and age. Patients undergoing more than one surgical procedure per year were significantly younger and had more deterioration of the quality of life aspects and mental health status compared with patients with less frequent surgical treatment sessions.

Unexpectedly, there was no association between voice quality as measured by the VHI and the number

Table II. Sample characteristics and descriptive statistics.

Characteristic	Value
Total no. of patients included	27
Age, total included	47 years (range 21–71, mean 46 ± 13)
Females	10/27, 37% (mean age 47 ± 13 years)
Males	17/27, 63% (mean age 46 ± 13 years)
Juvenile onset	7/27, 26%
Adult onset	20/27, 74%
HPV6	17/27, 63%
HPV11	2/27, 7%
HPV16	2/27, 7%
HPV31	1/27, 4%
Low treatment frequency, surgery <1 per year	15/27, 56%
High treatment frequency, surgery >1 per year	12/27, 44%
Papilloma located in glottis area	22/27, 81%
Papilloma located in supraglottic area	1/27, 4%
Papilloma located in supraglottic and glottic area	2/27, 7%
Papilloma located in transglottic area	1/27, 4%

The patients were evaluated according to the frequency of surgery by using a cut-off point of one treatment per year.

of surgical sessions performed. The majority of patients were HPV6-positive (63%). Patients with high-risk HPV subtypes rated their situation significantly worse in several subscales, including social functioning, emotional limitation and general mental health. The high-risk HPV subtypes may reflect different biological HPV entities and a generally more aggressive state of disease with significant impact on quality life and voice, as opposed to the low-risk HPV subtypes. Females revealed significantly lower scores regarding the domains of physical functioning, physical role limitation, body pain and general health, and also the overall physical health status as compared with males.

The northern Sweden RRP data set-up is limited by the low numbers of patients with high-risk HPV subtypes, thus not allowing major significant conclusions or clinical guidelines, instead reflecting tendencies. Furthermore, the patient material was restricted to a territorial referral centre for RRP in northern Sweden and compared to Swedish normative values. Since this is not a frequent disease, there were relatively few observations for some of the disease categories, such as juvenile RRP and high-risk HPV, limiting the possibilities to analyse relative risks for these groups. The use of statistics comparing historical normative values with our data is a second limitation since there are no data on the variation of the normative values for VHI and SF-36 over time.

A third and important limitation in the study design was that there was no premorbid assessment of patient functional vocal ability. However, the findings still illustrate some trends from a clear-cut reduced social ability in the RRP patients; there seemed to be little impact on quality of life or voice handicap as compared with normative values for the Swedish population. This might be due to a small number of observations and chance or possibly a robust ability to adjust to the disease. The socially reduced ability as a single value reflects the impact on quality of life and alters the patients' social relationships and interactions.

In the present study, a selected RRP group represented by low age, females, high-risk HPV subtypes and high numbers of treatment sessions has been identified as more vulnerable when measured in terms of quality of voice and quality of life.

Conclusion

The majority of the patients with RRP experience a significant self-reported voice dysfunction. A selected RRP group represented by low age, females, high-risk HPV subtypes and high levels of treatment sessions seem to be more vulnerable for morbidity in terms of quality of voice and quality of life. However, more studies are warranted to identify if this is really the case. There is a moderate impact of RRP on voice, but in some domains (social) there is also significant impact on quality of life. Optimization of the treatment plans for these patients should include prophylactic vaccines and initial information strategies.

Acknowledgments

This investigation was supported by grants from Lion's Cancer Research Foundation at Umeå University, LP 1945, 2012 and LP 1998, 2013 and Umeå University Medical Faculty, Sweden. We would like to thank Professor Karin Nylander for the critical analysis of the manuscript.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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II

Incidence of tonsillar cancer in northern Sweden: Impact of human papilloma virus

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Received October 16, 2014; Accepted July 16, 2015

DOI: 10.3892/ol.2015.3775

Abstract. The incidence rate of tonsillar cancer is increasing worldwide. The current study identifies a parallel increase in the incidence of tonsillar cancer, human papilloma virus (HPV) and p16 expression among a population from northern Sweden, a sparsely populated area, confirming the strong association between p16 and HPV infection in tonsillar tissue. Data from the Swedish Cancer Registry was assessed to identify cases of tonsillar cancer in the northern territorial area of Sweden. HPV DNA was extracted from paraffin embedded diagnostic biopsies and detected by polymerase chain reaction using general primers Gp5+/6+ and CpI/IIG. Expression of p16 was identified by immunochemistry. Patients were grouped into urban or rural residence categories. A total of 214 cases were identified, comprising 155 (72.4%) men and 59 (27.6%) women, and 65 of these patients, who presented between 2000 and 2012, were analyzed. The overall median age for the analyzed patients was 58 years; 48 (74%) were males (median age, 57.5 years) and 17 (26%) were females (median age, 65 years). Of the 65 specimens, 59 (91%) were positive for HPV, and 62 (95%) expressed p16. The incidence of tonsillar cancer in the cohort demonstrated a 2-fold increase between 1990 and 2013; specifically, a 2.7-fold increase was observed in men whilst the female group exhibited only a small increase. These findings demonstrate a strong association between p16 expression and HPV infection in tonsillar malignancies. The incidence of HPV-positive tonsillar cancer has increased in recent years, even in sparsely populated regions, as demonstrated in northern Sweden.

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Key words: head and neck cancer, human papilloma virus, immunohistochemistry, incidence, p16, tonsillar cancer

Introduction

Squamous cell carcinoma of the head and neck (HNSCC) is the sixth most common malignancy worldwide, with ~650,000 new cases diagnosed each year (1). These tumors may originate in the oral cavity, oropharynx, hypopharynx or larynx (2). The incidence of HNSCC in Sweden is ~1,200 new cases per year (2), which accounts for 3-4% of all new cancer diagnoses in the country. Worldwide, the incidence is two times higher in men than in women (3).

Oropharyngeal cancer develops in the base of the tongue and palatine tonsils, the posterior pharyngeal wall and the soft palate. Established predisposing factors include heavy tobacco smoking and alcohol consumption, which appear to act synergistically (4). Tonsillar cancer is the most common form of oropharyngeal cancer in Sweden (3). Typical symptoms include swallowing difficulties, unilateral pain in the throat and ear, and lumps in the neck. The incidence of tonsillar cancer appears to have been increasing during recent years, despite a decline in smoking and alcohol consumption. One possible factor contributing to this may be mucosal infection with human papilloma virus (HPV) (5-8).

It has been proposed that HPV-positive tonsillar carcinomas should be considered different tumor entities from HPV-negative tonsillar carcinomas, as HPV-positive tumors arise in the tonsillar crypts while the non-HPV-associated form originates from the tonsillar surface (9). The majority of HPV-positive tonsillar cancers are associated with the high-risk HPV type 16, and this HPV type may be present in ~95% of the HPV-positive cases, whilst other types, such as 18, 31, 33 and 35, are relatively infrequently associated with HNSCC (10).

Radiotherapy is the optimal and standard treatment option at present, and may be combined with chemotherapy in more advanced cases. To date, curative surgical monotherapy of tonsillar cancer has been unsuccessful. However, recent results using transoral robotic surgery as single modality treatment have demonstrated a potential therapeutic benefit (11,12). Certain reports have suggested that HPV-related malignancies have a more favorable prognosis, regardless of the chosen treatment strategy (13-15).

Currently, HPV is the most common sexually transmitted disease; however, even the most sensitive DNA tests may fail to detect infections caused by the virus (16). The recent introduction of vaccination against HPV 16-associated anogenital cancers in non-infected subjects has demonstrated a promising protective effect through the prevention of infection. This approach may have a role in the prevention of specific HPV-subtype-positive head and neck malignancies in the future (17). The use of HPV vaccines as preventive and therapeutic treatments has been discussed in the literature (18,19).

The aim of the current study was first to assess the incidence of tonsillar cancer in northern Sweden, one of western Europe's most sparsely populated regions. Secondly, the study aimed to address the recent incidence in this region compared with reported incidences in densely populated regions. It further aimed to assess the proportion of HPV-associated tonsillar cancers and its surrogate marker, p16, and finally, to determine whether there were variations in the manifestation of the disease associated with urban versus rural areas in the region.

Materials and methods

Patients. The Regional Ethical Review Board of Umeå University (Umeå, Sweden) approved the analysis of paraffin-embedded retrospective samples from the Biobank North (County Council of Västerbotten, Västerbotten, Sweden; approval nos. 2012-276-32M and 2010-277-31M). In addition, study was conducted in accordance with the Declaration of Helsinki (20).

This retrospective observational study included all consenting patients diagnosed with tonsillar cancer between 1990 and 2013 at the University Hospital of Umeå (Umeå, Sweden). In order to identify cases of tonsillar neoplasms, information was extracted from the Swedish Cancer Registry database (www.socialstyrelsen.se/register/halsodataregister/cancerregistret/inenglish) using the International Classification of Diseases (ICD)-7 code 145.0. The designation 'northern Sweden' was defined as the part of Sweden consisting of the counties Västerbotten, Norrbotten, Västernorrland and Jämtland, which include a total population of 882,563 and ~4 inhabitants per square kilometer. For comparative purposes, data from the Swedish Cancer Registry database regarding the whole Swedish population of ~9.5 million was used (2013).

Pre-treatment tumor samples were collected by biopsy or surgical resection. Paraffin-embedded tumor blocks were retrieved from the archive of the Department of Laboratory Medicine/Pathology at the University Hospital of Umeå. The clinical characteristics of the study population are summarized in Table I.

HPV detection by polymerase chain reaction (PCR). DNA was extracted from paraffin-embedded diagnostic biopsies using a QIAamp DNA FFPE Tissue Kit or QIAamp Mini Kit (Qiagen, Inc., Valencia, CA, USA), according to the manufacturer's instructions. A general HPV PCR analysis was run using 100 ng of extracted DNA from each patient and the general primers GP5+/6+, as previously described (21). The primers were as follows: 5'-TTT GTT ACT GTG GTA GAT ACT AC-3' for GP5+ and 5'-GAA AAA TAA ACT GTA AAT CAT ATT C-3' for GP6+. The 50 μl PCR mixture consisted of 5 μl GeneAmp 10X PCR Gold Buffer, 200 μM of each dNTP (GeneAmp dNTP mix), 3.5 mM $MgCl_2$ (all from Applied Biosystems Life Technologies, Foster City, CA, USA), 25 pmol of each primer and 1 unit of AmpliTaq Gold DNA Polymerase (Applied Biosystems Life Technologies).

Amplification was performed in a Biometra Professional Thermocycler (Thermo Fisher Scientific, Waltham, MA, USA) and was initiated with denaturation for 4 min at 94˚C, followed by 40 amplification cycles of denaturation at 94˚C for 1 min, annealing at 44˚C for 1 min and elongation at 72˚C for 2 min. The final cycle ended with a prolonged elongation step at 72˚C for 10 min. PCR products were run on a 2.5% agarose gel (SeaKem® LE Agarose; Lonza, Rockland, ME, USA) in 0.5X Tris/Borate/EDTA-buffer (TBE; 1 L of 10X TBE: 121.1 g tris base, 46 g boric acid and 7.44 g EDTA made up to 1 L with distilled water), stained with 0.5X GelRed (Biotium, Hayward, CA, USA), and visualized under UV-light. Fragments of 130-150 bp were considered positive.

To avoid false negative results due to a disrupted L1 gene, negative samples were run with the general primers CpI/IIG, as described in Smits *et al* (22). The primers were as follows: 5'-TTA TCW TAT GCC CAY TGT ACC AT-3' for CpI and 5'-ATG TTA ATW SAG CCW CCA AAA TT-3' for CpIIG. Briefly, the PCR mixture consisted of 5 μl GeneAmp 10X PCR Gold Buffer, 200 μM of each dNTP (GeneAmp dNTP mix), 3 mM $MgCl_2$, 17 pmol CpI, 26 pmol CpIIG, 0.5 μl bovine serum albumin and 1 unit of AmpliTaq Gold DNA Polymerase. The amplification consisted of denaturation for 5 min at 94˚C, followed by 40 amplification cycles of denaturation at 95˚C for 1 min, annealing at 55˚C for 1 min and elongation at 72˚C for 2 min. The final cycle ended with a prolonged elongation step at 72˚C for 4 min. Fragments were analyzed as described for the previous PCR analysis, and products of 188 bp were considered positive.

PCR with the GP5+/6+ primers was run at least twice. In cases with weak or divergent results, the PCR was repeated. Additionally, in random cases, a new DNA preparation was used to validate the method.

p16 immunohistochemistry and scoring system. For the detection of p16, staining was performed in a Ventana staining machine (BenchMark ULTRA; Ventana Medical Systems, Tuscon, AZ, USA) according to the supplier's recommendations. An antibody against p16 (monoclonal mouse anti-human; cat. no. sc-56330) from Santa Cruz Biotechnology (Dallas, TX, USA) was used at a dilution of 1:200. Prior to staining, slides were pretreated in Tris-EDTA (10 mM Tris-HCl, 1 mM disodium EDTA; pH 8.0). The antibody was visualized using the Ultraview Universal DAB Detection Kit (Ventana Medical Systems) and staining was observed using a light microscope (BX51; Olympus Corporation, Tokyo, Japan).

The quickscore system was used for scoring the percentage of tumor cells expressing p16 and intensity of staining (23). The proportion of tumor cells expressing p16 was graded from 1-6: 1, 0-4%; 2, 5-19%; 3, 20-39%; 4, 40-59%; 5, 60-79%; or 6, 80-100%. Staining intensity in turn was divided into 4 grades: 0, negative; 1, weak; 2, intermediate; and 3, strong. By multiplying the score for percentage of tumor cells

Table I. Clinical characteristics of the patients included in the HPV DNA and p16 analysis.

Specimen number	Year of diagnosis	% tumor cells in sample	p16 quickscore	HPV	Gender	Age at diagnosis, years
1	2012	25[a]	6	1	F	67
2	2012	70[a]	12	1	M	64
3	2012	70[a]	12	1	M	53
4	2012	50[a]	12	1	M	51
6	2011	80[a]	12	1	M	70
8	2012	40[a]	12	1	M	53
9	2011	60[a]	12	1	M	55
10	2011	30[a]	12	1	M	54
12	2011	30[a]	12	1	M	45
13	2011	70[a]	12	1	F	65
14	2011	70[a]	12	1	M	72
15	2012	80[a]	12	1	F	76
16	2012	60[a]	12	1	F	87
17	2012	95[a]	6	1	F	65
18	2010	70[a]	18	1	M	58
19	2010	20[a]	12	1	F	75
20	2010	75[a]	12	1	M	59
21	2010	80[a]	12	1	M	70
23	2010	70[a]	12	1	M	71
24	2010	50[a]	12	1	M	49
25	2006	60[a]	18	1	M	48
26	2006	80[a]	12	1	M	55
27	2006	60[a]	12	1	F	76
28	2007	60[a]	0	0	M	51
29	2007	95[a]	12	1	M	58
30	2007	5[a]/10[b]	12	1	M	48
31	2007	60[a]	12	1	M	64
33	2006	60[a]	12	1	M	59
34	2006	75[a]	12	1	M	60
35	2005	80[a]	12	1	M	62
36	2005	80[a]	12	1	M	47
37	2005	90[a]	2	0	F	64
38	2005	75[a]	12	1	M	58
39	2003	80[a]	12	1	M	57
40	2002	20[a]	18	1	M	61
41	2002	80[a]	12	1	F	64
42	2001	50[a]	0	0	F	53
43	2001	70[a]	12	1	F	46
44	2001	80[a]	5	1	M	58
45	2000	70[a]	12	1	F	69
46	2000	85[a]	12	1	F	63
47	2000	30[a]	18	1	M	52
48	2001	60[a]	12	1	F	68
49	2001	5[a]	12	1	F	45
50	2004	60[a]	2	0	F	54
51	2009	95[a]	12	1	M	48
52	2009	80[a]	12	1	M	53
54	2009	40[a]	12	1	M	51
55	2001	30[a]	12	1	M	74
56	2004	40[a]	4	0	M	73
57	2008	80[a]	12	1	M	60

Table I. Continued.

Specimen number	Year of diagnosis	% tumor cells in sample	p16 quickscore	HPV	Gender	Age at diagnosis, years
58	2008	70[a]	12	1	M	49
59	2008	80[a]	12	1	F	53
60	2009	70[a]	18	1	M	63
61	2008	10[a]/50[b]	12	1	M	56
62	2009	40[a]	12	1	M	63
63	2009	5[a]/15[b]	12	1	M	60
64	2009	50[a]	12	1	M	49
66	2009	60[a]	12	0	M	63
68	2008	60[a]	18	1	M	53
69	2008	70[a]	12	1	M	51
70	2008	60[a]	18	1	M	53
71	2010	60[a]	0	1	M	66
73	2012	70[a]	12	1	M	62
74	2002	20[a]	12	1	M	56

[a]Before and [b]after sectioning. p16 quickscore = intensity score [0 (negative), 1 (weak), 2 (intermediate) or 3 (strong)] x proportion score [1 (0-4%), 2 (5-19%), 3 (20-39%), 4 (40-59%), 5 (60-79%) or 6 (80-100%)]. HPV: 0, negative; 1, positive. M, male; F, female; HPV, human papilloma virus.

expressing the protein by the intensity score, a quickscore ranging from 0-18 was determined.

Classification according to place of residence. The code numbers of the biopsy samples were used to identify the personal identification number of each patient and, subsequently, the residence address was extracted through the patients' electronic records system (Systeam Cross; Eskilstuna, Sweden). Subjects were placed into either urban or rural groups depending on the location of their private residence. A Swedish urban area was defined as a residential area with ≥200 inhabitants where the distance between buildings is <200 meters (24), whilst a rural address was one where the number of local inhabitants was <200 and the distance between houses was ≥200 meters (25).

Analysis and statistics. The total age-standardized incidence of tonsillar cancer over the period from 1990-2013 was determined using northern Sweden's standard population (2000) and data obtained from the Swedish Cancer Registry. Data regarding the cases of head and neck cancer was extracted from the Swedish Cancer Registry for comparative purposes (2). The Mann-Whitney U test was used to compare patient ages between different genders and HPV-positive and HPV-negative patients. The χ^2 test was used to investigate any differences in HPV-status between genders. Statistical analysis was performed using SPSS software version 22.0 (IBM SPSS, Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Incidence of HNSCC. From the period between 1990 and 2012, a total of 22,640 cases of head and neck cancer were identified in the Swedish Cancer Registry database, including tumors located in the hypopharynx, larynx, oropharynx, oral cavity, tongue, lips, nasopharynx, nose, paranasal sinuses and middle ear. Of these, 15,563 (68.7%) were men and 7,077 (31.3%) women. Cases from 2013 were not yet registered. The total number of cases of head and neck cancer in northern

Table II. Incidence of tonsillar cancer in northern Sweden per 100,000 individuals, according to the population of northern Sweden in 2000.

	Period				
Group	1990-94	1995-99	2000-04	2005-04	2010-13
Male	0.826	0.968	1.13	1.88	2.25
Female	0.46	0.488	0.564	0.494	0.482
Total	0.692	0.724	0.83	0.998	1.379

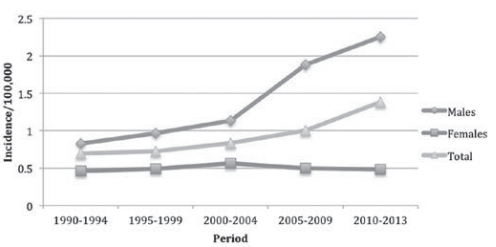

Figure 1. Incidence of tonsillar cancer in northern Sweden, 1990-2013. A 2-fold overall increase was observed; a 2.7-fold increase was observed in males while the female group demonstrated only a small increase.

Sweden during the same period was 1,978 (9% of all cases in Sweden). Of these patients, 1,297 (65.6%) were men and 681 (34.4%) were women.

The number of cases of tonsillar cancer (ICD-7 code 145.0) in northern Sweden between 1990 and 2013 according to the Swedish Cancer Registry was 214, comprising 155 (72.4%) men and 59 (27.6%) women.

The total age-standardized incidence of tonsillar cancer in northern Sweden doubled between 1990 and 2013, from 0.69-1.38 per 100,000 individuals. The increase in incidence in men was 2.7-fold (0.83-2.25 per 100,000), while the female incidence increased from 0.46 (1990) to 0.48 (2013) per 100,000 (Table II; Fig. 1).

HPV analysis. A total of 74 biopsy specimens from patients with tonsillar cancer between 2000 and 2012 were identified and obtained from the archives of the Department of Pathology, University Hospital of Umeå. Of these, 4 specimens were excluded as they contained too little or a complete lack of tumor tissue. Additionally, 5 samples were excluded as they were duplicates of patients already included in the database.

Of the 65 remaining specimens (median age, 58 years; mean age, 59.3 years; range, 45-87 years), 48 (74%) were males (median age, 57.5 years; mean, 57.6 years; range, 45-74 years) and 17 (26%) were females (median age, 65 years; mean, 64.1 years; range, 45-87 years). Age was significantly higher among female subjects compared with male subjects (U test, P=0.016).

HPV DNA was detected in 59 (91%) of the 65 biopsy specimens, while the remaining 6 samples (9%) were HPV-negative. There was no difference when comparing the variable of age between the HPV-positive and HPV-negative samples (P=0.856, U test). A χ^2 test identified no association between HPV-status and gender (P=0.179). In the HPV-positive cohort, there was a male dominance [3:1; 45 males (76%) vs. 14 females (24%)], whilst an equal proportion of males and females (1:1) was observed in the HPV-negative cohort. When comparing gender and age in the HPV-positive cohort, age was significantly higher in females compared with in males (U test, P=0.006).

p16 expression. Expression of p16 was scored in all 65 samples, with 62 samples (95%) positive for the p16 marker and 3 (5%) negative (Fig 2). Of the p16-positive samples, 7 (11%) received the highest score of 18 points, 49 (79%) a score of 12 (intermediate intensity) and 6 samples (10%) received 2-6 points.

Classification according to residence. The patients' residence area at the time of diagnosis indicated that the vast majority (64 patients, 98.5%) were living in urban areas, while only 1 patient (1.5%) resided in a rural area. This difference is due to the overall populations of these areas.

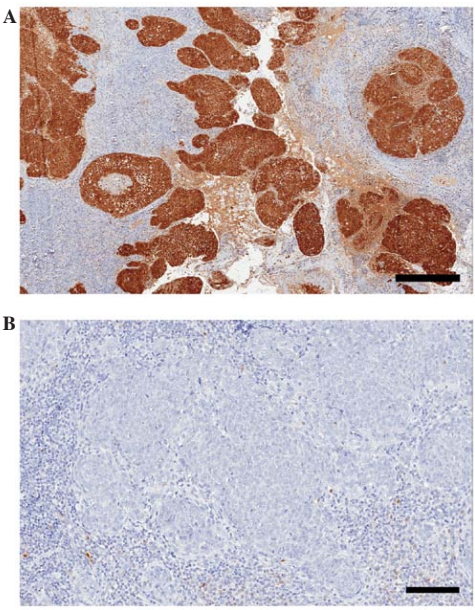

Figure 2. (A) Positive and (B) negative p16 staining in tonsillar squamous cell carcinoma. Scale bar, A, 400 μm; B, 100 μm. Stain, p16. (Quickscore, A, 12; B; 0).

Discussion

The aims of the current study were to investigate the proportion of p16-positive cases of HPV-related tonsillar cancer, to study the incidence of tonsillar cancer in the sparsely populated region of northern Sweden, and to examine whether this increasing trend is parallel to the incidence observed in other, more densely populated areas.

A 2-fold increase in cases of HPV-positive tonsillar cancer was observed during the analyzed period of 23-years (1990-2013), preferentially in male patients. Hammarstedt *et al* (5) reported a fold increase in tonsillar cancer incidence in the urban region of Stockholm of 2.8 (2.6 in men and 3.5 in women), while smoking had decreased in Sweden during the same period (1970-2002) (26). In the current findings, the increased incidence of tonsillar cancer was observed only in the male group. The reason for this disparity is unclear, although the present study included sampling from a more recent period of 11 years. Another factor may be the relatively recent knowledge and treatment patterns related to HPV 16 as a causative agent of cervical cancer.

The findings demonstrating no recent change in the incidence of tonsillar cancer in women in this region may be an indication of the differences in HPV exposure and susceptibility between sparsely populated and densely populated areas. This issue has not been examined to date. The increased incidence of HPV-related tonsillar cancer in males has been described in a study examining ethnic perspectives (27), in which a recent decline in incidence rates of tonsillar cancer was also observed for both white and black females aged 40-64 years in the USA. Hence, HPV infection in the oropharynx may be an explanation for at least part of the increase in the incidence of tonsillar cancer in men. Another possible explanation may be the traditional risk

factors of alcohol and smoking found in the male population in northern Sweden, which could have a synergistic effect with the HPV virus. At present, <20% of Swedish men smoke tobacco. The significantly lower mean age of the males in the current sample and, in particular, the lower mean age of the male HPV-related cases, may be an indicator of an altered sexual behavior and consequent frequent HPV contamination among men in northern Sweden.

Hammarstedt *et al* (28) reported that patients with HPV-positive tonsillar malignancies were younger (median age, 55 years) compared with patients with HPV-negative disease, and no similar difference regarding other oral cancers could be identified. This finding could not be confirmed in the current data due to the limited number of cases of HPV-negative tonsillar cancer.

The present results revealed that 91% of the cases of tonsillar cancer were HPV-positive. A similarly high proportion of HPV-positive cases of tonsillar cancer in Sweden has been reported by Näsman *et al* (29), who identified an increase from 68% (2000-2002) to 93% (2006-2007) (29). In general, HPV DNA has been reported to be detected in 45-100% of tonsillar cancer cases (30). It is possible that the cohorts with a lower proportion of HPV-positive cases are less recent, and this could indicate that the numbers of HPV-related tonsillar cancer are increased in more current published data due to the more reliable diagnostic methods available.

Differences in HPV-related tonsillar malignancies have been observed not only between urban and rural areas in Sweden, but also in other countries. Blomberg *et al* (7) described an increased incidence rate of HPV related tonsillar cancer between 1978 and 2007 in Denmark, while the incidence of non-HPV-associated HNSCC decreased in men, but remained unchanged in the female group. Another study of patterns of tonsillar cancer in South-eastern England, comparing the years 1987 and 2006, revealed an incidence of tonsillar cancer increasing from 0.600-1.45 per 100,000 among men (40-59 years), a lower age at diagnosis and an increase in median survival time (8). Similar studies have been performed in the USA and Finland, demonstrating a rising incidence of tonsillar cancer during the last decades (27,31).

Overexpression of p16 was observed in the present tumor specimens, confirming the results of a number of previous studies (4,32-36), as well as demonstrating the strong association between p16 and HPV-infection in tonsillar carcinomas. The majority of these specimens exhibited p16 expression of medium intensity, while a further 11% showed strong p16 expression.

In the last decade, a number of studies have reported the prognostic value of HPV and p16 analysis in HNSCC in general and in tonsillar cancer in particular. It has been confirmed that patients with HPV-associated tumors in the tonsillar region have a better prognosis compared with patients with HPV-negative tumors in terms of locoregional control and overall survival, regardless of treatment modality (4,14,15). Nichols *et al* (13) reported that patients with HPV-related tumors are younger and have smaller primary-site tumors and a more favorable survival rates compared with those with HPV-negative tumors. The overexpression of p16 is highly associated with HPV infection, and this may be used clinically as a surrogate marker and a prognostic guide of the disease. Large and often cystic lymph node involvement, along with clinically advanced stage (III-IV), are also associated with HPV-positive tumors (35,37).

The favorable prognosis associated with HPV positivity in tonsillar cancers has been studied by Lindquist *et al* (35), who reported an increased disease-specific survival rate in patients with HPV-associated tonsillar cancer (81%) compared with patients with HPV-negative tumors (36%), regardless of age, gender or tumor stage. These findings have been further supported by Fischer *et al* (36), who reported that the 5-year survival rate of patients with p16-positive oropharyngeal cancer of stages III-IV was nearly as good as for those with tumors of stages I-II. The prognostic value of p16 in the outcome of HPV-positive tonsillar neoplasms was also addressed. Cases with p16-positive tumors in the advanced stages (III-IV) had a 5-year survival of 54.1%, compared to 18% for those with p16-negative tumors. Regardless of tumor stage, p16-positive cases had a 5-year survival rate of 59.3% compared to 24.5% for the p16-negative cases (36). Results from the Danish DAHANCA 5 study revealed that p16-positive HNSCCs were associated with improved locoregional tumor control (58% vs. 28%) and increased disease-specific (72% vs. 34%) and overall (62% vs. 26%) survival rates compared with p16-negative tumors (38). The detection of p16 and HPV may therefore be an important tool as part of a comprehensive strategy to develop personalized treatment for tonsillar cancer.

Despite these findings, many current recommendations for the treatment of HPV-positive tonsillar tumors do not differ from those for HPV-negative tumors (39). Certain investigators have questioned the need for aggressive treatments for patients with HPV-positive tonsillar cancer, which has now been found to be associated with a better prognosis and is often observed in younger and healthier groups (4,39). The risks and benefits of aggressive chemoradiotherapy and surgery must be considered carefully. Current research is now focused on the de-escalation of treatment modalities for the HPV-related cases and emphasizes the introduction of a number of prognostic markers in order to identify such patients in the pre-treatment stage (4,38).

At present, there is a lack of clear evidence regarding the biological basis behind the differences in survival and prognosis between HPV-associated and HPV-negative malignancies. A possible explanation may be that the different molecular profile in patients with HPV- and p16-positive tumors constitutes a distinct subset of tonsillar malignancies (34). In addition, it is also reasonable to speculate that a younger, non-smoking cohort is associated with a reduced rate of co-morbidity and, therefore, improved non-cancer-related outcomes.

The route of transmission of HPV infection in oral carcinomas remains unclear. Much speculation has occurred regarding possible sexual transmission. The increasing evidence of tonsillar cancer during the last decades has been associated with a significant change in sexual habits, and HPV is currently considered to be the most common sexually transmitted infection (16). This issue has been studied by Hemminki *et al* (40), with results indicating that the husbands of patients with cervical cancer have a higher risk of tonsillar and tongue-based malignancies. Hence, the prophylactic

vaccination of women may also lead to decreased rates of oral HPV infection in the male population. The numbers of HPV infections in the oral cavity and, subsequently, HPV-associated tonsillar tumors are expected to surpass the rate of cervical malignancies by 2020, and this fact highlights the importance of vaccinating both genders in the future as a preventive measure against tonsillar cancer (41).

In summary, the present study identified incidences of HPV-positive and p16-expressing tonsillar cancers in men from northern Sweden that were similar to those already reported for Swedish urban populations, as well as populations outside of Sweden. This study may provide a basis for future projects investigating the prognosis of tonsillar carcinomas. Further investigation into the biological mechanisms of the improved survival and prognosis associated with the HPV-positive tonsillar cancers is warranted. Furthermore, it may be beneficial to evaluate the role and effect of HPV vaccination as a prophylactic measure against tonsillar and oral cancer, and the necessity for introducing vaccination strategies to the male population.

Acknowledgements

This study was supported by grants from Lion's Cancer Research Foundation (13-1998), at Umeå University Sweden.

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Acta Oto-Laryngologica. 2015; Early Online, 1–7

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ORIGINAL ARTICLE

Recurrent respiratory papillomatosis in northern Sweden: Clinical characteristics and practical guidance

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Abstract

Conclusion: Recurrent respiratory papillomatosis (RRP) patients with high surgical treatment frequency (≥ 1/year, HF) were significantly younger and had a more widespread laryngeal disease compared to a low frequency treated group (< 1 treatment/year, LF). This study confirms the existence of a clinical RRP group, not primarily related to HPV sub-type, but more care-intensive and in need of more vigilant follow-up. *Objectives:* RRP is associated with high morbidity due to its influence on breathing and voice. The purpose of this study was to characterize RRP patients in northern Sweden and investigate possible predictor factors affecting therapeutic needs. *Method:* Patients from the regional referral area (northern Sweden) were categorized for age, disease duration, juvenile or adult onset, profile of disease development, number of surgical sessions in relation to disease duration, laryngeal deposition of papilloma, gender, and HPV sub-types, in order to identify patients with increased need for frequent surgical treatment. *Results:* The median age of the RRP patients ($n = 48$) was 44.5 years; 34 (71%) were males and 14 (29%) females, most were infected with HPV 6. Patients with high surgical treatment frequency/year were significantly younger and showed more widespread papillomatous vegetation in the larynx, compared to the low frequency treated group.

Keywords: *Human papilloma virus, recurrent respiratory papillomatosis, HPV vaccine, tonsillar cancer, gender, airway surgery, data analysis*

Introduction

Recurrent respiratory papillomatosis (RRP) is a neoplastic infectious disease caused by specific sub-types of human papilloma virus (HPV). The incidence in the US is estimated at 4.3 per 100,000 children and 1.8 per 100,000 adults [1]. The HPV virus infects the squamous epithelium of the skin (HPV 1, 2, and 4), the genital mucosa (condyloma acuminata, HPV 6, and 11), and the upper airways (recurrent respiratory papillomatosis, HPV 6, or 11). Some HPV sub-types are classified as oncogenic (for example HPV 16, 18, 31, 33) and are implicated in the development of malignant tumours such as cervical and oropharyngeal cancer. In total, close to 200 sub-types are recognized today [2].

RRP is characterized by benign, wart-like lesions causing hoarseness and airway obstruction. The disease accounts for extensive morbidity due to its influence on breathing. The voice is affected, and patients usually have a lifelong need for recurrent surgical treatment sessions. Patients with RRP are usually

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(Received 16 December 2014; accepted 20 January 2015)

ISSN 0001-6489 print/ISSN 1651-2251 online
DOI: 10.3109/00016489.2015.1048378

infected with low-risk HPV limited to the larynx [3], although pulmonary metastasis of HPV benign tumours has been described [4]. Some reports describe asymptomatic genital infection with HPV 16 [5], as well as asymptomatic carriers of HPV 6 in the larynx [6].

The introduction of prophylactic vaccination against HPV-associated anogenital cancers and genital warts in non-infected subjects has revealed promising preventative results with 95–100% efficacy [7]. When results become available on the efficiency of these vaccines against HPV-related head and neck cancer, it is likely that an even greater benefit from these vaccines will be seen [8]. The HPV vaccination of already infected patients has been described as being alternatively effective [9] and ineffective [10]. There seems to be a lower efficacy for the vaccine in preventing HPV 6 and 11 infections compared its effect on high-risk HPV virus (HPV 16 and 18) [7].

HPV is presumed to be transmitted vertically, with intra-partum or perinatal transmission between mother and child [11]. Trials in pregnant women have been conducted in order to prevent vertical transmission of HPV infection, although results have not yet been enough to provide clinical guidance in this context [12]. HPV infection is reported to be the most common sexually transmitted disease worldwide [3].

No single therapeutic modality reliably eradicates the HPV virus. The treatment options currently available aim at minimizing symptoms by reducing the viral load through surgical excision using a CO_2 laser, microdebrider, or radiofrequency ablation ('coblation'). The optimal goal is to prevent infection, and then for those who become infected surgery should be as simple and atraumatic as possible. The outcome of surgical treatment sessions depends on different factors like surgical skill, availability of surgical techniques, but also most likely on unmeasured factors such as patient immune competence, viral load, presence of viral co-infections, and other mechanisms [13]. Adjuvant treatment options have been used in well-defined sub-groups of RRP [14]. Our hope is that the mapping of RRP patients in northern Sweden will affect the pre-operative information we provide to our patients, concerning both the surgical treatment and also the current prophylactic vaccination strategy and practical therapeutic vaccination evaluation.

The aim of this study was 2-fold: first, to assess clinical characteristics and possible predictor factors affecting the therapeutic needs of RRP patients in northern Sweden, and, second, to identify a potentially high-risk RRP group that could require more vigilant follow-up and also be suitable for evaluation of the therapeutic effects of HPV vaccination.

Materials and methods

Participants

The study was approved by the Regional Ethical Review Board in Umeå (2012-276-32m, 2010-277-31m). All patients gave their informed consent to participate in this prospective cohort study. The study included all patients diagnosed or treated with recurrent respiratory papillomatosis in the public healthcare system of the four northernmost counties of Sweden, between May 2006 and August 2014. None of the responders had been vaccinated with HPV vaccines, underwent surgery with microdebrider or radiofrequency ablation, or received adjuvant therapies such as a-interferon, indole-3-carbinol and cidofovir during the last 10 years.

A total of 48 patients were included in the study and underwent diagnosis and treatment at the tertiary referral centre for the region, the University Hospital of Umeå. The Department of Otorhinolaryngology at this hospital provides RRP patient services (inquiry and surgical treatment) to a population of ~ 930,000 inhabitants. The sample size was determined by access in the region to RRP patients.

Patients were categorized (Table I) for age, disease duration, disease onset (childhood/juvenile under the age of 5 years and adult period), disease development, number of surgical sessions in relation to disease duration, laryngeal spread of papilloma (glottic-, sub- and supraglottic location), gender, and HPV sub-types according to a locally modified Derkay staging assessment system [15]. Data were stored in the northern Sweden RRP patient database. The main indications for surgery were hoarseness and mild airway obstruction (mild stridor at activity). Surgery was performed using an AcuPulse Lumenis CO2 laser, under general anaesthesia. One of the authors (KO) performed all therapeutic interventions.

Remission was defined as a voice asymptomatic period for > 1 month. Young age was defined as below 40 years of age. The necessity for surgery was based on the subjective severity of symptoms, and this decision followed a detailed discussion with the patient. Patients were divided into low-frequency (LF, < 1 surgical treatment/year) and high-frequency (HF, ≥ 1 surgical treatment session/year) groups. None of the patients smoked. Occurrence of allergy, gastroesophageal reflux disease (GERD), and asthma were not reason for exclusion. None of the juvenile RRP patients were premature at birth.

Table I. Sample characteristics.

	Median	*n* (%)
Total number, $n = 48$		
Females	—	14 (29%)
Males	—	34 (71%)
Age, females	48.0 years	—
Age, males	43.5 years	—
Juvenile onset	—	6 (12%)
Adult onset	—	42 (88%)
HPV 6	—	32 (67%)
HPV 11	—	5 (10%)
HPV 16	—	2 (4%)
HPV 31	—	1 (2%)
Not determined HPV sub-type	—	8 (17%)
Low frequency < 1/year; median *n* surgery/year	0.4	—
High frequency ≥ 1/year; median *n* surgery/year	1.6	—
Glottic papilloma	—	48 (100%)
Supraglottic papilloma, total	—	7 (15)
Subglottic papilloma, total	—	5 (10%)
Complete remission	46	46 (96%)
Non-complete remission	2	2 (4%)
Visual sub-clinical vocal fold web	—	5 (10%)
Cancer development	—	1 (2%)

Remission = asymptomatic periods of > 1 month.

Subjects and assessments

All patients were evaluated pre- and ~ 8 weeks post-operatively and as needed afterwards. Examinations were performed using an Olympus ENF P4 transnasal flexible fibre-optic endoscope. The stroboscopic equipment was a Wolf type 5052 and the camera was a Wolf endocam 5502. The video stroboscopic examination was recorded and stored in Picsara, an audiovisual documentation system used at the hospital. The following tasks were evaluated during the endoscopic examination of the larynx: sustained/i/ and/m/as close to habitual speaking pitch and intensity as possible. Intra-operatively, biopsies were obtained for histopathological studies and HPV identification. A papilloma tissue sample was kept in sterile saline and sent to the Clinical Microbiology Laboratory at Skåne University Hospital in Malmö for analysis. Polymerase chain reaction products were analysed for specific genotypes using the Luminex system containing type-specific probes for mucosal HPV types (high-risk 16,18,31,33 types and low-risk 6 and 11 types). The laboratory is accredited according to the ISO15189 standard for 14 oncogenic types and for HPV6 and HPV11 used in the HPV test. The Luminex assay also included two broadly reactive 'universal' probes and samples positive only for this universal probe were typed by sequencing.

Data analysis

Pairwise comparison between two sets of data was obtained under the same conditions, given an expected normal distribution and variance which motivated the use of Student *t*-test. If the normality assumption was judged to be inappropriate, the Wilcoxon signed-rank test was used. All significance testing was performed at the 0.05 level; two sided *p*-values were reported. When justified, the normal distribution was investigated using the Kolmogorov-Smirnov test. The statistical analysis was evaluated with caution due to limited groups constituting the study population.

Results

Subject characteristics

The median age of the RRP patients by August 2014 ($n = 48$) was 44.5 years; 34 (71%) were males (median age = 43.5 years, mean = 41.2 years) and 14 (29%) were females (median age = 48.0 years, mean = 44 years). The median age at RRP diagnosis was 32 years (range = 1–60 years, SD = 15.4 years). The median duration of RRP disease was 7.2 years (females = 6.5 years; males = 8 years). The number of operations during the diagnosed period of illness in median was in the group with ≥ 1 treatment/year (HF) 1.6 surgical sessions, vs 0.4 surgical sessions in the group with low treatment frequency < 1/year (LF). All patients were evaluated pre- and ~ 8 weeks post-operatively.

Surgical sessions and gender characterization

Results are presented in Table II. The main findings are the following. The majority of RRP patients was male (71%), belonging to the low-frequency treatment group (65%) and was infected with HPV 6 (67%), presented with glottic papilloma distribution (100%) and had an adult onset (94%). Even if the number of vaccinated subjects was low, there was a trend towards less surgery in this group after HPV vaccination. When comparing the groups of high treatment frequency (HF) with low frequency of treatment (LF), lower age was seen in the HF group (42 vs 47 years, $p = 0.02$, *t*-test). Supra- and subglottic spread in the larynx was higher in the HF group,

Table II. Frequency of surgical sessions and gender characterization in a northern Sweden RRP survey.

	High frequency (HF)		Low frequency (LF)			Females (F)		Males (M)		
	n	% HF	*n*	% LF	*p*-value, *t*-test	*n*	% F	*n*	% M	*p*-value
Age	Median age 37		Median age 46		0.04	Median age 48.0		Median age 43.5		NS
Number of surgery/year	Median 1.6		Median 0.4		0.02	Median 0.4		Median 0.5		NS
Number (48*)	17	35	31	65		14	29	34	71	
HPV 6 (32*)	13	76	19	61	NS	6	42	26	76	NS
HPV 11 (5*)	2	12	3	9	NS	3	21	2	6	NS
HPV 16 (2*)	1	6	1	3	NS	1	7	1	3	NS
HPV 31 (1*)	0	—	1	3	NS	1	7	0	—	NS
HPV not settled (8*)	1	6	7	23	0.02	3	21	5	15	NS
Glottic papilloma total (48*)	17	100	31	100	NS	14	100	34	100	NS
Supraglottic papilloma (7*)	5	29	2	6	0.03	2	14	5	15	NS
Subglottic papilloma (5*)	5	29	0	0	0.02	1	7	4	12	NS
Juvenile-onset (6*)	3	17	3	9	NS	2	14	4	12	NS
Adult-onset (42*)	13	76	29	94	0.002	11	78	31	91	NS
Web (5*)	3	17	2	6	NS	1	7	4	12	NS
Vaccine programme (8*)	7	41	1	3	NS ($p = 0.07$)	2	14	6	17	NS

(*) Total number. All significant testing was performed at the 0.05 level.
NS, non-significant.

compared with LF ($p = 0.03$ and 0.02, respectively, *t*-test). More patients in the LF group had an adult RRP-onset ($p = 0.002$, *t*-test) and a larger number in the HF cohort chose to be vaccinated ($p = 0.07$, *t*-test). No correlation between surgical complications (web development), specific HPV sub-types, state of remission, and frequency of surgical sessions was seen (HF vs LF). Apart from the significant male dominance in the northern Sweden RRP survey there was no significant difference in gender aspects. The necessity for surgery was generally based on the subjective severity of symptoms, followed by a detailed discussion with the patient.

HPV vaccination

Eight (two females and six males) out of 48 (16%) patients chose to be included in a three-step vaccination procedure (Gardasil, Merck & Co., USA). Seven of these patients belonged to the HF-group, and all had been informed about the scientific lack of other than prophylactic effects. Even if the number of vaccinated subjects was low, there was a trend towards less surgery in this group after HPV vaccination. We choose to reflect one case longitudinally in reducing the treatment intervals due to heterogeneous follow-up periods after vaccination at the group level (Figure 1).

HPV sub-populations

Two RRP patients (4%) were diagnosed with high-risk HPV 16 and one of those developed a glottic malignancy 3 years after diagnosis of dysplasia and 1.5 years after infection with HPV 16 had been confirmed. The patient was successfully treated with radiotherapy as a single modality treatment (2011) according to national standard. The patient was still (at the time of writing this text) in remission phase for papilloma as well as malignancy. In two of the eight patients with incomplete HPV sub-type, clinical presence of RRP was unquestionable, even if biopsies were negative for HPV sub-type procedure. The RRP diagnosis was based on clinical stroboscopic findings of exophytic vocal fold wart-like lesions and histopathology.

Six patients out of 48 (12%) had juvenile disease onset (before the age of 5 years), four boys and two girls (median age = 2.7 years). Two patients (4%) showed incomplete remission between surgical sessions measured subjectively as well as objectively.

Discussion

This is the first prospective cohort study of a well-defined RRP population living in northern Sweden

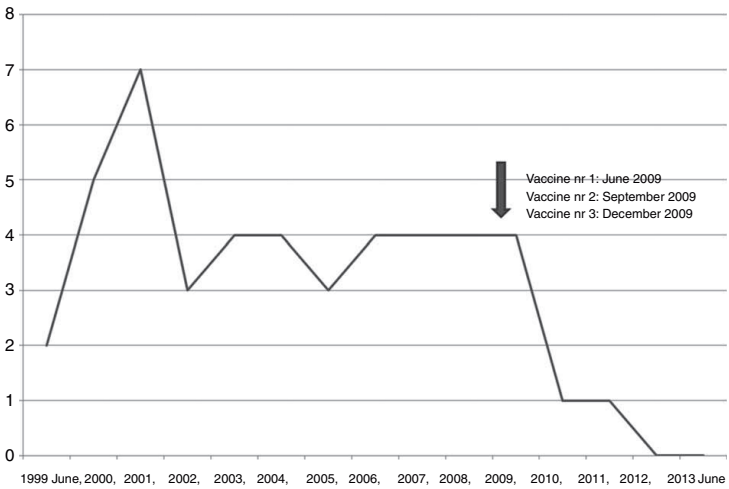

Figure 1. One vaccinated RRP subject, number of surgical treatment sessions per year before and after 3-step vaccination procedure with quadrivalent HPV vaccination (Gardasil).

investigating a number of disease-related factors and the need of surgical intervention. Reflecting the aim of our study we conclude that we both have characterized the northern region RRP patients and have identified a care intensive sub-group of RRP patients not primarily related to HPV sub-type but to treatment intensity. In contrast to Derkay et al. [15], the goal was not to streamline the prediction of treatment intervals based on anatomical and symptom score, but rather to identify associations of the other factors to the observations of the HPV deposit in the larynx. No Derkay score was therefore calculated.

All patients had a histopathologic diagnosis, medical history, and clinical findings (endoscopic examination) compatible with RRP; none of them was typed for HPV status when admitted to the tertiary referral University hospital of Umeå. The majority of RRP patients was male, treated less than once a year, infected with HPV 6, had a glottis distribution, and an adult onset. The predominance of HPV 6 in the airway is in line with the results by Komloš et al. [16]. The demographic description of RRP patients was divergent when analysing the frequency of treatment. RRP patients with high surgical treatment frequency/year (≥ 1, HF) were significantly younger and had a more widespread laryngeal disease compared to the low frequency treated group (< 1 treatment/year, LF). Young age (Table II) is regarded superior to the high-risk sub-type when prioritizing a patient cohort in need of more vigilant follow-up, though, however, not reducing the need of clinical monitoring of glottal squamous dysplasia and/or carcinoma *in situ* histopathology associated with the sub-types of HPV, 16, and 18. It is likely that the younger population in the highly productive professional years is more care demanding based on voice-loaded jobs requiring high levels of functionality.

The need for an optimized and above all a targeted treatment of RRP patients is potentiated by the fact that RRP is the second most common cause of hoarseness in children. The disease is characterized by extensive morbidity due to lifelong, repetitive influence on breathing, followed by the need of recurrent symptomatic, but not curative surgical sessions. In a previous study, we evaluated the quality of voice and life in 27 out of 48 consecutive RRP patients included in this study, and found that patients in need of surgical treatment ≥ 1/year were significantly younger. An impairment of voice quality was also noticed as compared with healthy individuals. Even the quality-of-life was inferior in some patients (females, patients with frequent surgical treatment sessions, and patients with high-risk HPV types) [17].

Due to the high levels of discomfort caused by extra surgical sessions, there were significantly more non-typed HPV in the LF group compared to the HF group. Although RRP is not usually associated with high-risk HPV [18], HPV 16 was found in two cases in the study (4%). Due to reliable methods for detection of HPV nucleic acids, there is currently enough clinical evidence that HPV-positive tonsillar carcinomas are different tumour entities compared to the non-HPV-associated form, which is associated with

the traditional risk factors of smoking and alcohol [19]. High-risk HPV represents a major cause for the increase in incidence of malignancies in a number of anatomical sites: in the upper aero-digestive tract, and mainly in the oropharynx. It is not known, however, if HPV-positive laryngeal carcinomas differ in a biological way compared to HPV-negative laryngeal cancer [20]. We propose that there is a possibility for such a partition not necessary related to HPV subtype but to other heretofore unknown or unrecognized factors.

The incidence of squamous cell carcinoma in the head and neck in Sweden is ~ 1200 new cases each year, accounting for 3–4% of all cancers in Sweden [21]. There is an increased incidence of tonsillar cancer in males vs females [19]. A male incidence polarization was also reproducible in our RRP material. This gender aspect is highly interesting in the light of the severity of the two diseases and the existing HPV vaccine programme in Sweden, which currently includes girls only with prevention against HPV 16-induced cervical cancer.

The incidence rate of RRP is still at an unjustifiably high level. Possibly, a modified and well-planned vaccination strategy for both sexes could in time reduce the rate of RRP. It is difficult to justify, from an ethical aspect, exclusion of the male population in this prevention. These findings support the idea of strong benefit of a three-step vaccine procedure in a selected group of RRP patients defined by low age, high frequency of treatment sessions, and widespread laryngeal papillomatosis. Although we did not observe a definitive association between high-risk HPV sub-types and high frequency treatment, there was still a strong suggestion that such an association can exist, although a much larger patient material will be needed to establish this. In our opinion, even with only preliminary evidence, there is an argument for treating high-risk HPV sub-types with such a vaccination treatment strategy, while the question is studied further.

Despite some study design limitations, including the relatively small regional population size, one can still appreciate that the incidence data for RRP and the need of clinical guidance of RRP patients in the catchment area makes the comparison between the HF and LF groups valid. In a future study, it would be desirable to compare and assess subject characteristics at specific time points in their illness, although the enrolment of the RRP patients with different disease trajectories did not allow such a study design. This regional HPV cohort could benefit from comparing the clinical course of the disease between different regions and in different healthcare systems, despite being a small cohort in size.

Conclusion

The majority of RRP patients in northern Sweden are men infected with HPV 6. RRP patients with high surgical treatment frequency (≥ 1, HF) seem to be younger and have a more widespread laryngeal disease as compared to the low frequency treated group (< 1 treatment/year, LF). We confirm the existence of a clinical RRP group, not related to the high-risk HPV sub-type, but still more care-intensive and in need of more vigilant follow-up.

Acknowledgements

This investigation was supported by grants from Lion's Cancer research Foundation at Umeå University, LP-13 1998 and LP-14 2048. We would like to thank Professor Michael Haney for the critical analysis on the linguistic aspects of the manuscript.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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IV

FULL PAPER

BJC

British Journal of Cancer (2015) 113, 321–326 | doi: 10.1038/bjc.2015.207

Keywords: squamous cell carcinoma; tongue; HPV; p16; syndecan-1

Expression of p16 in squamous cell carcinoma of the mobile tongue is independent of HPV infection despite presence of the HPV-receptor syndecan-1

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Background: Tongue squamous cell carcinoma (TSCC) is increasing in incidence, especially among young patients and preferably females. Infection with human papilloma virus (HPV) has been suggested as a cause of SCC in the head and neck, and the proportion of oropharyngeal cancers caused by HPV has steadily increased.

Methods: Samples from 109 patients with primary TSCC were analysed for the presence of HPV16 by *in situ* hybridisation and for expression of its surrogate marker p16 and the HPV receptor syndecan-1 by immunhistochemistry.

Results: No evidence of HPV16 DNA was observed in the tumours, although one-third showed p16 staining. There was no difference in the expression of the primary HPV receptor, syndecan-1, between TSCC and a group of tonsil SCC.

Conclusion: Whereas p16 is expressed in some TSCCs, HPV16 is undetectable, therefore, p16 cannot be used as a surrogate marker for high-risk HPV-infection in this tumour. Despite presence of the HPV-receptor syndecan-1 in TSCC, HPV prefers the tonsillar environment. Lack of p16 associates with worse prognosis primarily in patients aged $\leqslant$40 years with tongue SCC. The improved prognosis seen in p16-positive TSCC can be due to induction of a senescent phenotype or an inherent radiosensitivity due to the ability of p16 to inhibit homologous recombination repair.

Squamous cell carcinoma of the head and neck (SCCHN) is a collective term for tumours of several different locations within the head and neck area showing widely varying histology dependent on location. Even within the limited area of the oral cavity, there are differences in the expression of proteins and miRNAs between sites that are seen also between tumour-free samples (Boldrup *et al*, 2011; Boldrup *et al*, 2012). The most commonly tumour affected site within the oral cavity is the tongue, and tongue squamous cell carcinoma (TSCC) is an increasing group of tumours especially among young patients (Hilly *et al*, 2013; Troeltzsch *et al*, 2014) and

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Received 17 March 2015; revised 7 May 2015; accepted 13 May 2015; published online 9 June 2015

preferably females (Patel *et al*, 2011). The reason for this increase is so far not known, even if it is clear that this age group, defined as being ⩽40 years, has not been exposed to the known risk factors for this disease, smoking and alcohol, for the same duration and extent as many of the older patients.

Recently, infection with human papilloma virus (HPV) has been suggested as a cause of SCCHN, especially among young patients, and the proportion of oropharyngeal cancers caused by HPV has steadily increased in recent years (Marur *et al*, 2010; Chaturvedi *et al*, 2011). Thus an increase in HPV infection incidence could possibly relate also to the increased incidence of TSCC in young patients. In order to infect an epithelial cell, HPV first has to bind to and then enter the cell (Rautava and Syrjänen, 2012). In this binding process, a heparin sulphate proteoglycan, syndecan-1, has been pinpointed as the primary receptor for HPV in keratinocytes (Shafti-Keramat *et al*, 2003). Infection seems to be restricted to tumour only, as HPV-positive oropharyngeal tumours show complete lack of active HPV around the tumour and also lack field cancerisation (Rietbergen, *et al*, 2014). These observations emphasise that HPV-positive and -negative tumours are two distinct groups, at least in the oropharyngeal area (Pannone *et al*, 2011), where HPV-associated tumours often present at higher clinical stage with advanced nodal disease, despite being of smaller size. Despite this advanced clinical stage, the prognosis and overall disease-free survival for patients suffering from these tumours are superior to that of patients with non-HPV-associated tumours (Shah *et al*, 2009).

There are several ways of analysing HPV infection available (Schlecht *et al*, 2011), including PCR and *in situ* hybridisation for specific HPV types, most commonly the high-risk-type HPV16. It has also been proposed that expression of p16 correlates with HPV infection. The tumour-suppressor p16 (also called Cdkn2a) is a cdk (cyclin-dependent kinase) inhibitor, which inhibits binding of cdks 4 and 6 to cyclin D1. This in turn inhibits phosphorylation of Rb, which is needed for release of E2F to enable entry into the cell cycle (Witkiewicz *et al*, 2011). Apart from being a tumour suppressor, p16 is also a surrogate marker for high-risk HPV infection and has been found to be upregulated in HPV-positive oropharyngeal cancers. HPV infection could lead to accumulation of p16 protein via targeting of Rb (Witkiewicz *et al*, 2011). p16 is expressed in a wide variety of SCCs, other than those originating from cervix, and seems to be a reliable surrogate marker for high-risk HPV also in oropharynx. However, p16 is not a specific marker of HPV status in non-oropharyngeal SCC (Doxtader and Katzenstein, 2012). In a small group of 25 young (<40 years) patients with TSCC, p16 positivity correlated with improved relapse-free survival (Harris *et al*, 2011). This finding is in accordance with another study of oral SCC where patients with cancer with lower p16 expression were more likely to develop a recurrence (Shah *et al*, 2009).

In this study, we clarified the clinical and prognostic importance of HPV 16 and p16 in a large group of SCC tumours in the mobile tongue. By comparing the expression of the HPV receptor syndecan-1 in a subgroup of these TSCC with a group of tonsillar cancers, we also wanted to clarify whether there is any difference in the expression of this receptor between these sites, which could explain the differences in incidence of HPV infection between them.

MATERIALS AND METHODS

Materials. Samples from 96 patients with primary TSCC and formalin-fixed, paraffin-embedded biopsies available at Clinical Pathology, Umeå University Hospital, Sweden, and 13 patients available at the Second University of Naples, Multidisciplinary Department of Medical, Surgical and Dental Specialties, Naples, Italy were included in the project. Both patient groups had been treated during a period of 15 years. Of the 109 patients, 54 were men and 55 were women with a mean age of 63.5 years, ranging from 19 to 93 years. Patients were grouped into three groups based on age at diagnosis: ⩽40, 41–65, and >65 years. For clinical data, see Table 1. The majority of patients (66%) had received preoperative radiotherapy followed by surgery and 31% were primarily treated with surgery.

The mean follow-up time was 45.5 months (ranging from 1 to 179 months). At the end of the study, survival was measured as: alive disease free, alive with disease, dead of disease, dead of other disease or dead with disease but not with oral cancer as first cause of death. Data on survival and cause of death were obtained from the clinical files or the Swedish Death Registry.

A group of 65 patients with tonsillar carcinoma (17 women and 48 men) were included in the analysis of syndecan-1 expression. The mean age within this group was 59.9 years (range 45–87). This group of tumours had been analysed previously for HPV presence and p16 expression (Loizou *et al*, 2015). The project was approved by the local Ethical Committee (dnr 03–201 and dnr 08-003M).

Immunohistochemistry. For detection of p16, the antibody (Santa Cruz Biotechnology, Dallas, TX, USA) was diluted 1 : 200. Slides were pretreated in Tris-EDTA pH 8.0, and staining was performed in a Ventana staining machine (Ventana Medical Sytems Inc, Roche, Tuscon, AZ, USA) according to the supplier's recommendations. Eighty-nine of the tongue tumours and 65 cases of tonsillar cancer were also stained with an antibody detecting syndecan-1 (Abcam, Cambridge, UK) diluted 1 : 100, after pretreatment in citrate buffer pH 6.0. Staining was performed in a Ventana staining machine.

Scoring. Samples were scored for proportion of tumour cells expressing p16 and syndecan-1 and for intensity of staining. Proportion of tumour cells expressing the proteins was divided into six stages, where 1 = 0–4%, 2 = 5–19%, 3 = 20–39%, 4 = 40–59%, 5 = 60–79% and 6 = 80–100%, and intensity in four stages, with 0 = negative, 1 = weak, 2 = intermediate and 3 = strong staining. By multiplying the percentage of tumour cells expressing the protein with intensity, a quick score (QS) ranging from 0 to 18 was

Table 1. Patient data, including age at diagnosis, gender and TNM stage

Age at diagnosis, years	Number	Male/female ratio	T1	T2	T3	T4	N0	N+	M0	M1	
⩽40	16 14.7%	7/9 1 : 1.3	2	10	2	2	12	4	16	0	16
41–65	38 34.9%	26/12 2.2 : 1	14	11	9	4	27	11	37	1	38
>65	55 50.4%	21/34 1 : 1.6	15	19	8	13	41	14	54	1	55
	109		31	40	19	19	80	29	107	2	109

Abbreviations: M = distant metastasis; N = nodal metastasis; T = tumour size.

obtained (Detre *et al*, 1995). The p16-stained slides were evaluated independently by three of the authors NS, KS and KN. Results were then compared, and cases of disagreement were discussed in a joint session. The syndecan-stained slides were evaluated by KN only.

HPV16 *in situ* hybridisation. *In situ* hybridisation was used to investigate the presence of HPV16 DNA in 71 of the samples (all 36 p16-positive and 35 p16-negative tumours). HPV16 plasmid DNA was obtained from ATCC (LGC Standards, Middlesex, UK), amplified and purified using HiPure Plasmid Maxi-prep Kit (Invitrogen, Paisley, UK). Plasmid DNA was labelled by nick-translation (Invitrogen) for 90 min at 14 °C in the presence of digoxigenin-16-dUTP (Roche, West Sussex, UK) and purified by repeated ethanol precipitation in the presence of 100 × excess of salmon sperm and Cot-1 DNA (Invitrogen). Sections were dewaxed, endogenous peroxidase activity was blocked in H_2O_2 in methanol and tissue digested with varying concentrations of proteinase K (Sigma) in 50 mM Tris pH 7.5 and 1 mM EDTA pH 8.0. Probe (1 ng μl^{-1}) in hybridisation buffer was applied, and sections were coverslipped before rapid high temperature mico-wave-mediated denaturation, as previously described (Coates *et al*, 1987; Coates *et al*, 1991). After overnight hybridisation at 42 °C, sections were washed twice in 2 × SCC at room temperature, twice in 0.1 × SCC at 45 °C and once in 2 × SCC at room temperature, each for 5 min. For immunoshistochemical detection, sections were incubated with mouse anti-digoxin (1/5000; Sigma) followed by biotinylated anti-mouse and avidin–biotin peroxidase complex (Elite ABC Kit, Vector Laboratories (Cambridgeshire, UK), used according to the manufacturer's instructions) and detection with an intensified DAB/imidazole reaction. Nuclei were lightly stained with haematoxylin, dehydrated, cleared and mounted in resin for light microscopy. A positive control section (cervix) was performed with each batch of tumours analysed.

Statistical analysis. SPSS version 22 (IBM Corporation, New York, NY, USA) was used for statistical analysis. QSs were correlated to clinical data. For calculation of *P*-values, Chi^2-test was used, and in survival analysis 2- and 5-year survival was used. A *P*-value <0.05 was considered statistically significant.

RESULTS

Clinical data. The majority of tumours were localised on the lateral border of the mobile tongue (67%), 20% on the ventral side and 2% on the dorsal side. In 11%, lesions were so widespread that it was not possible to state the prime localisation of the lesion on the mobile tongue. There was a statistically significant correlation between patients suffering from an extended lesion showing both lower survival rate and disease-free status ($P=0.009$ for 2-year survival, 0.027 for 5-year survival and 0.022 for status). Patients in the young age group (⩽40 years) showed lower survival and disease-free rate compared with the older age patients (>65 years). Gender did not affect survival. A statistically significant correlation was seen between T and staging and survival rate and disease-free condition, with decreased survival with increased T ($P=0.000$ for 2-year survival, 0.002 for 5-year survival and 0.001 for status) and higher stage ($P=0.000$ for 2-year survival, 0.010 for 5-year survival and 0.003 for status). There was also a statistically significant correlation between node positive, N+, tumours and poor 2-year survival rate ($P=0.040$; Table 2).

Immunohistochemistry

p16. Of the 109 tumour samples, 73 (67%) were negative for the presence of p16 independent of site of lesion. Weak expression (defined as a QS of 1–5) was seen in 19%, and a QS of 6–18 in 14% (Table 3 and Figure 1). Comparing p16 expression between age groups, 75% of tumours in patients aged ⩽40 years were p16 negative, compared with 66% within the other two age groups (Table 3). At the 5-year follow-up, 4 of the 12 p16-negative young patients (33%) were alive, compared with 62% of the patients aged 41–65 years having passed 5-year follow-up.

Of the 29 node positive, N+, tumours, 52% were p16 negative. No correlation was seen between p16 and localisation of the lesion, age, gender, grading or relapse.

Syndecan. All 89 tongue SCCs analysed for syndecan expression were positive, with 82% having a QS of 6–18. There was no statistically significant correlation between expression of p16 and syndecan-1. Most patients with a QS of 6–18, 65%, were N0. Similar results were seen for patients with tonsillar carcinoma, with all tumours expressing the receptor, and the majority, 74%, having a QS of 6–18 (Table 4 and Figure 2).

HPV16 *in situ* hybridisation. In the 71 samples analysed, including all 36 p16-positive samples, no evidence of HPV16 DNA was observed in the tumour tissue. The technique used is able to detect low copy number viral DNA (approximately two copies of HPV DNA per cell; Coates *et al*, 1991; Herrington *et al*, 1991), and a positive control of human cervical epithelium with histological evidence of productive infection showed the presence of a hybridisation signal throughout the epithelium, including basal cells that contain only a few viral DNA copies per cell.

DISCUSSION

Squamous cell carcinoma of the head and neck remains a significant problem and is the eighth most common cause of cancer death worldwide. The aetiology of epithelial cancers of the head and neck is considered to be a multifactorial, sequential process. Several factors are involved in oral carcinogenesis, such as age, gender, ethnicity, lifestyle, genetic background, status of health and exposure to one or more oncogenic factors. The two major lifestyle risk factors in SCCHN are tobacco use and alcohol; however, 15–20% of patients do not have any known tobacco or alcohol exposure (Patel *et al*, 2011). The disease incidence has also been seen increasing among younger patients who often lack these traditional risk factors, and one unique subgroup of patients

Table 2. Patient outcome at end of the study, with female:male ratio given in parenthesis

	Status (female:male)		2-year survival			5-year survival					
Age at diagnosis, years	ADF/DAD	AWD/DOD/DWD	Yes	No	Not passed	Yes	No	Not passed	Relapse	Never free of tumour	
⩽40	5 (2 : 3)	11 (7 : 4)	8	8	0	5	11	0	11	0	16
41–65	25 (5 : 20)	13 (7 : 6)	26	12	0	19	13	6	8	6	38
>65	23 (13 : 10)	32 (21 : 11)	25	26	4	13	28	14	10	19	55
	53	56	59	46	4	37	52	20	29	25	109

Abbreviations: ADF = alive, disease-free, AWD = alive with disease, DAD = dead of other disease; DOD = dead of disease, DWD = dead with disease but not with oral cancer as the first cause of death.

Table 3. p16 status in relation to patient age, N status and 2- and 5-year survival, respectively

p16 QS	Patients aged ⩽40 years	Patients aged 41–65 years	Patients aged >65 years	N0	N+	2-year survival	5-year survival	
0	12 (75%)	25 (66%)	36 (66%)	58 (72%)	15 (52%)	44 (75%)	28 (76%)	73 (67%)
1–5	2 (12.5%)	9 (24%)	10 (18%)	15 (19%)	6 (21%)	9 (15%)	5 (13%)	21 (19%)
6–18	2 (12.5%)	4 (10%)	9 (16%)	7 (9%)	8 (27%)	6 (10%)	4 (11%)	15 (14%)
Total	16	38	55	80	29	59/105	37/89	109

Abbreviation: N = nodal metastasis; QS = quick score. Concerning 2-year survival, 4 patients had not been followed that long, and in the analysis of 5-year survival, 20 patients had too short follow-up.

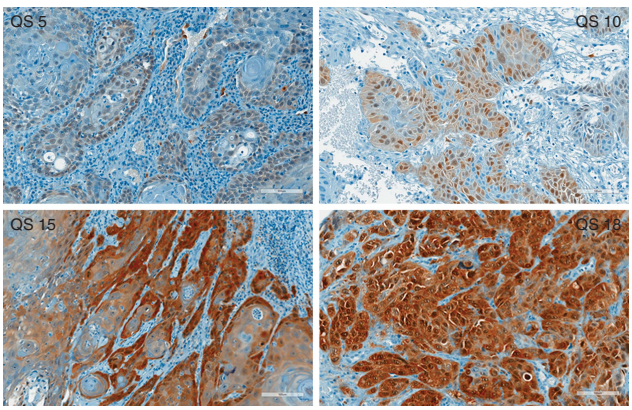

Figure 1. p16 expressing tongue SCC with a QS of 5, 10, 15 and 18 respectively.

Table 4. Syndecan status in relation to age of patients with tongue and tonsillar SCC, respectively

	Tongue SCC			Tonsil SCC		
Syndecan QS	⩽40 years	41–65 years	>65 years	⩽40 years	41–65 years	>65 years
0	0	0	0	—	0	0
1–5	3	5	8	—	14	3
6–18	11	29	33	—	36	12
Total	14	34	41	—	50	15

Abbreviation: QS = quick score; SCC, squamous cell carcinoma.

identified is young patients with TSCC. The tongue is the most common site for oral cancer development, and TSCC is more aggressive than other SCCs of the oral cavity, with properties of rapid local invasion and high regional relapse rate. TSCCs also show a more split invasive growth pattern and a more intense inflammatory response at the tumour interface compared with the whole group of SCCHN tumours (Lundqvist *et al*, 2012).

TSCCs may not genomically differ when comparing young and older patients (Pickering *et al*, 2014), and it has been hypothesised that the increasing incidence of SCCHN in young patients is related to infection with high-risk subtypes of the HPV. In normal oral mucosa, the incidence of HPV infection is very low (Migaldi *et al*, 2012), whereas a recent meta-analysis showed a strong association between HPV and oral potentially malignant lesions and oral carcinoma (Syrjanen *et al*, 2011). HPV-positive tumours are generally found in the oropharynx and have been associated with younger patients who are less likely to be smokers or drinkers and show improved response to therapy and overall survival (Pannone *et al*, 2011; Sand and Jalouli, 2014). The proportion of SCCHN that are potentially HPV related (cancers of the tongue base and the Waldeyer's ring) increased in the past 30 years, perhaps as a result of changing sexual behaviours, and nowadays about 18% of oropharyngeal cancers and >90% of tonsillar cancers worldwide are HPV associated (Pannone *et al*, 2011; Loizou *et al*, 2015).

We have previously not been able to detect HPV in TSCCs using PCR and Luminex, whereas 91% of tonsillar carcinomas were HPV positive using the same PCR method (Loizou *et al*, 2015). Based on the clinical impact of mapping HPV status in oropharyngeal SCC (Pannone *et al*, 2011), we were encouraged to go further in this analysis and used here *in situ* hybridisation for detection of HPV16. However, no virus could be detected in TSCCs using this highly sensitive method, capable of detecting very low viral copy numbers in both experimental situations and clinical samples (Coates *et al*, 1991; Herrington *et al*, 1991). These data therefore suggest that HPV16 is either not present or is present at extremely low levels in the majority of p16-positive TSCCs, including those arising in young patients. However, due to the variable fixation and processing of these clinical samples, which influences the sensitivity of *in situ* hybridisation, we cannot completely exclude the presence of HPV16, and it is also possible that other high-risk HPV types are present, although other studies consistently demonstrate that HPV16 is the most prevalent type found in the oral cavity (Chaturvedi *et al*, 2011; Schlecht *et al*, 2011).

Molecular profiling of HPV-positive tumours has shown them to be commonly associated with p16 overexpression, whereas tumours not associated with HPV are seldom p16 positive. The lack of p16 expression defines a subgroup of oropharyngeal cancer patients with increased risk of local recurrence and worse clinical outcome (Shah *et al*, 2009). p16 protein overexpression has thus been proposed as a surrogate marker of HPV infection even if

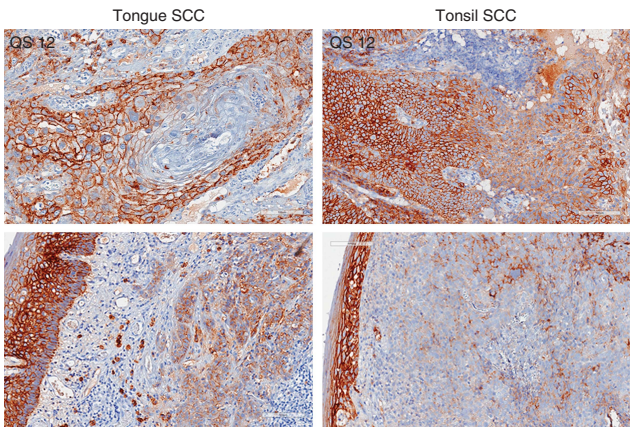

Figure 2. Expression of syndecan in tongue SCC, left panel, and tonsillar SCC, right panel. The two photos at the bottom show intense syndecan staining in surface epithelium in contrast to considerably weaker expression in tumour cells in the connective tissue.

not all studies confirm its prognostic significance in SCCHN (Gröbe *et al*, 2013). In our group of tongue tumours, 67% were p16 negative. Considering age, 75% of young patients were p16 negative compared with the other age groups where 66% of the tumours were p16 negative. Looking at follow-up for the p16-negative young patients, the majority (67%) was either still suffering from tumour or dead of or with disease. Even if differences are not statistically significant (the group of young patients is limited), results are in accordance with the previously shown worse outcome for p16-negative tumours (Shah *et al*, 2009) and also in line with the study of Harris *et al* (2011) showing the importance of p16 as a prognostic marker. Our results are further in concordance with the worse prognosis shown for young patients with TSCC (Lundqvist *et al*, 2012). The cases showing p16 expression in the absence of detectable HPV could in turn be explained by infection with other HPV types (Jordan *et al*, 2012), other unidentified infectious agents (Harris *et al*, 2011), or molecular alterations in the p16 pathway independent of infection with high-risk HPV, which may include transcriptional upregulation by oncogenic transcription factors such as Ets and Myc, alterations of Ras-MAPK pathways or loss of Rb (reviewed in Li *et al*, 2011; Romagosa *et al*, 2011; Witkiewicz *et al*, 2011). Indeed, high-level expression of p16 in the absence of HPV is well recognised outside of oropharyngeal and cervical cancer (Doxtader and Katzenstein, 2012; Hoffmann *et al*, 2012; Bussu *et al*, 2013). Thus, although unrelated to HPV, p16 expression in tongue SCC reflects an oncogenic process that is different from p16-negative cancers and provides an improved prognosis.

Another interesting finding was that expression of the primary receptor for HPV, syndecan-1, did not differ between these tongue SCC and a group of tonsillar SCC with a high percentage, 91%, of HPV infection (Loizou *et al*, 2015). This indicates that conditions for entering the tissue are fairly similar between tongue and tonsil, at least considering receptor availability, yet the virus seems to prefer the tonsillar environment. Recently, there has been a discussion on the impact of co-infection with various viruses, for example, HSV and EBV, where the latter preferably infects cells in a lymphocytic environment. Results are, however, not conclusive (Sand and Jalouli, 2014), still it is tempting to speculate that an explanation for the absence of HPV infection seen in tongue SCC could be lack of viral collaboration.

Taken together, we have shown that HPV16 is undetectable in tongue SCC. It can also be concluded that p16 cannot be used as a surrogate marker for HPV infection in tongue SCC, at least not when using the methods currently at hand. Looking at the prime receptor for HPV, syndecan-1, conditions for enabling entrance in the tissue are the same in the tongue as in the tonsil that gives room for speculation on the potential value of co-infection with other viruses or factors more common in the tonsillar area.

In concert with HPV-positive OSCC showing overall better outcome than HPV-negative oral cancers (Pannone *et al*, 2011), we suggest that lack of p16 expression in TSCC is an indicator of worse prognosis primarily in young patients suffering from this devastating disease. Although the mechanism(s) for improved prognosis in p16-positive TSCC is unclear, it may relate to either the induction of a senescent phenotype and thereby slow tumour growth (Ramagosa *et al*, 2011; Witkiewicz *et al*, 2011) or to an inherent radiosensitivity due to impaired DNA double-strand break repair capacity (Rieckmann *et al*, 2013), which in turn may relate to the ability of p16 to directly inhibit homologous recombination repair in HPV-positive SCCHN (Dok *et al*, 2014).

ACKNOWLEDGEMENTS

This study was supported by grants from Lion's Cancer Research Foundation, LP 13–1997 and LP 13–1998, Umeå University, the Swedish Cancer Society Contract number 14 0752, Västerbotten County Council and RECAMO; CZ 1.05/2.1.00/03.0101 grant LO1413.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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