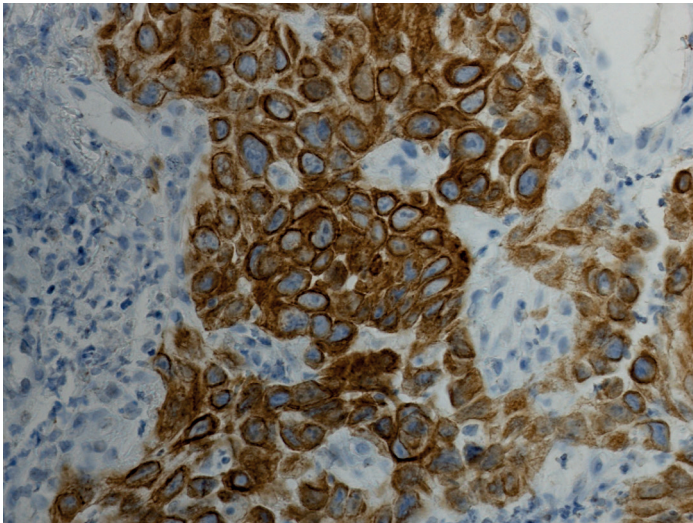


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**Oropharyngeal Cancer: Changing Management
and the Role of Toll-like Receptors**



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OROPHARYNGEAL CANCER: CHANGING MANAGEMENT AND THE ROLE OF TOLL-LIKE RECEPTORS

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ACADEMIC DISSERTATION

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To Susanna, Kaarlo, and
our unborn child

The pessimist complains about the
wind; the optimist expects it to change;
the realist adjusts the sails.
William Arthur Ward

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1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

- I. Jouhi L, Halme E, Irjala H, Saarilahti K, Koivunen P, Pukkila M, Hagström J, Haglund C, Lindholm P, Hirvikoski P, Vaittinen S, Ellonen A, Tikanto J, Blomster H, Laranne J, Grénman R, Mäkitie A, Atula T, Epidemiological and treatment-related factors contribute to improved outcome of oropharyngeal squamous cell carcinoma in Finland. *Acta Oncol.* 2017;17:1-11 [Epub ahead of print]
- II. Jouhi L, Atula T, Saarilahti K, Hagström J, Haglund C, Mohamed H, Mäkitie A, Keski-Säntti H. Changing trends in the management of the neck in oropharyngeal squamous cell carcinoma. *Head Neck.* 2017;39(7):1412-20.
- III. Jouhi L, Datta N, Renkonen S, Atula T, Mäkitie A, Haglund C, Ahmed A, Syrjänen S, Grénman R, Auvinen E, Lehtonen S, Hagström J. Expression of toll-like receptors in HPV-positive and HPV-negative oropharyngeal squamous cell carcinoma--an in vivo and in vitro study. *Tumour Biol.* 2015;36(10):7755-64.
- IV. Jouhi L, Mohamed H, Mäkitie A, Remes SM, Haglund C, Atula T*, Hagström J*. Toll-like receptor 5 and 7 expression may impact prognosis of HPV-positive oropharyngeal squamous cell carcinoma patients. *Cancer Immunol Immunother.* 2017;66(12):1619-1629

*Equal contribution

The publications are referred in the text to their roman numerals. These original publications have been reprinted with the permission of the copyright holders.

2. ABBREVIATIONS

| | |
|---------|---|
| 3D-CRT | 3D-conformal chemoradiotherapy |
| AKT | serine/threonine protein kinase B |
| BNCR | Boron neutron capture therapy |
| cD1 | Cyclin D1 |
| CDK | Cyclin dependent kinase |
| cNo | No clinically evident regional lymph node metastases |
| cN+ | Clinically evident regional lymph node metastases |
| CRT | Chemoradiotherapy |
| CT | Chemotherapy |
| CUP | Carcinoma of unknown primary |
| DAMP | Danger-associated molecular pattern |
| DFS | Disease-free survival |
| DSS | Disease-specific survival |
| ENE | Extranodal extension |
| FDG-PET | ¹⁸ F-fluoro-2-deoxy-2-d-glucose positron emission tomography |
| FNAC | Fine-needle aspiration cytology |
| Gy | Gray |
| H&E | Hematoxyline-eosine |
| HNSCC | Head and neck squamous cell carcinoma |
| HPV | Human papillomavirus |
| HR | High risk |
| IHC | Immunohistochemistry |
| IMPT | Intensity-modulated proton therapy |
| IMRT | Intensity-modulated radiotherapy |
| ISH | <i>In situ</i> hybridization |
| LR | Low risk |
| MLC | Multiple leaf collimator |
| MRI | Magnetic resonance imaging |
| N+ | Presence of regional metastasis (nodal metastasis) |
| ND | Neck dissection |
| NF-κB | Nuclear factor κB |
| OAR | Organ at risk |
| OP | Oropharynx |
| OPSCC | Oropharyngeal squamous cell carcinoma |
| OS | Overall survival |
| OTSCC | Oral tongue squamous cell carcinoma |
| p16 | p16 ^{INK4A} |
| PAMP | Pathogen-associated molecular pattern |
| PCR | Polymerase chain reaction |
| pRB | Retinoblastoma |
| RFS | Recurrence-free survival |

| | |
|------|---------------------------|
| RR | Regional recurrence |
| RT | Radiotherapy |
| SCC | Squamous cell carcinoma |
| TLR | Toll-like receptor |
| TMA | Tissue microarray |
| TNM | Tumor node metastasis |
| TORS | Transoral robotic surgery |

3. ABSTRACT

Head and neck cancers constitute the seventh most common cancer group worldwide. Their incidence has been declining in the Western world, along with the decrease in tobacco smoking. However, the incidence of oropharyngeal squamous cell carcinoma (OPSCC) has been rising over the last two to three decades in many Western countries. This trend is attributed to human papillomavirus (HPV), which is responsible for the majority of OPSCC cases, whereas, according to most reports, the incidence of HPV-unrelated OPSCC has decreased. If the current increasing trend continues, the incidence of OPSCC will surpass that of HPV-induced cervical carcinoma by the year 2020.

HPV-positive OPSCC differs from the HPV-negative form in various aspects: Patients typically have less tobacco and alcohol consumption. They tend to have smaller primary tumors but more advanced disease in the neck. In addition, their tumors usually have a higher histopathological grade, and several other biological differences, such as immunohistochemically detectable protein p16^{INK4A} (p16) overexpression reflecting HPV positivity.

Treatment for OPSCC typically includes either surgery and postoperative oncological treatment or definitive oncological treatment. Along with the increased incidence of OPSCC, the treatment modalities have also been changing towards a more non-surgical (organ-preserving) approach, later in this thesis referred to as an oncological approach, and the use of upfront surgery has been decreasing. In addition, according to some national protocols, the use of definitive oncological treatment, such as concurrent chemoradiotherapy (CRT), should be the principal method in OPSCC treatment, whereas surgery should be reserved for salvage purposes only. However, in some countries surgery has a more important role in OPSCC treatment.

The increase in the proportion of patients carrying a HPV-positive tumor has created a demand to change treatment protocols, because within this patient group the response to CRT is typically markedly better than in patients carrying a HPV-unrelated tumor. Moreover, the long-term effects of the treatment may significantly reduce the patients' quality of life. Patients with HPV-positive tumors are generally younger and they have better odds of survival, but their post-treatment lifetime may be harmed by major treatment-related morbidity. Because of this, patients who carry a HPV-positive tumor, and are never-smokers, and have low T and N classes, may benefit from treatment de-escalation. Many prospective randomized trials delivering de-

intensified treatment for this OPSCC patient group with the lowest risk of disease recurrence are currently ongoing.

Validated predictive biomarkers could aid in the treatment individualization of OPSCC. Currently, many biomarkers besides HPV are suggested to be prognostic in OPSCC. However, the only well-validated prognostic biomarkers in OPSCC are HPV and protein p16.

As OPSCC that carries an HPV association has an inflammatory background, innate immunity may play a significant role in disease etiology. Toll-like receptors (TLRs) are receptors, which recognize endogenous and exogenous molecular patterns. Their activation results in inflammation. In HPV-associated cervical carcinoma, alteration in the expression of TLRs has been observed. These receptors are also expressed in an altered pattern when HPV infection persists in the cervix. Therefore, these receptors could have a role in HPV-associated OPSCC.

The first part of the present study analyzed the treatment and outcome in two patient series treated over a ten-year period. Study I included all OPSCC patients treated at the Finnish university hospitals, and Study II focused on the management of the neck in cN+ disease of patients treated at the Helsinki University Hospital. Study I gathered population-based information on the treatment, outcome, and factors affecting the prognosis of OPSCC in Finland during the years from 2000 to 2009, when the treatment protocol was changing towards a more oncological approach. The cohort included 674 patients, and during the study period, the incidence of this disease increased, which mainly occurred in the group of patients carrying a HPV-associated tumor. The outcome of lateral-wall OPSCC (tonsil) remained similar compared to an earlier Finnish nationwide report, but the outcome of anterior-wall disease (base of tongue) slightly improved. The factors contributing to an improved outcome in our patient cohort included female sex, p16 positivity, early T and N classes, and intensity-modulated radiotherapy (IMRT).

The presence of neck metastasis has a significant effect on survival, and treatment of the neck remains one of the key issues in the management of head and neck cancer patients. Survival after regional recurrence is poor in OPSCC. Therefore, Study II analyzed the treatment provided for cN+ disease in a series of 201 patients. The relative rate of neck dissections (NDs) had decreased while the delivery of definitive CRT had increased. However, the regional control rate had not worsened. Regional failures occurred in patients with class cN2b or higher and they often developed on the contralateral side of the neck, including in patients with an ipsilateral primary tumor. Thus, according to our results, bilateral neck treatment is warranted in all patients having metastases in the neck in order to prevent regional failures.

The second part of this thesis study (Studies III and IV) evaluated the role of TLRs in OPSCC. As a pilot study, we analyzed TLR 2, 3, 4, 5, 7, and 9 expression, p16 expression, and HPV status in 35 OPSCC samples. TLR 5, 7, and 9 expression varied according to p16 and HPV status. Among HPV-positive tumors, TLR 5 and 9 were less expressed, and TLR 7 was more expressed. Cell-culture studies on OPSCC and oral tongue squamous cell carcinoma cell lines revealed similar results. Based on these results, we evaluated the association of TLR 5, 7, and 9 with clinicopathological and outcome data in a cohort of 202 OPSCC patients, which provided further support for our previous results. In addition, the findings indicated that high TLR 5 expression and low TLR 7 expression were related to poor disease-specific survival in the group of HPV-positive OPSCC patients.

4. SUMMARY IN FINNISH

Pään ja kaulan syövät ovat maailman seitsemänneksi yleisin syöpätyyppi. Niiden esiintyvyys länsimaissa on laskenut tupakoinnin vähentyessä. Suunielusyöpä on kuitenkin yleistynyt monessa länsimaassa viimeisen muutaman vuosikymmenen aikana. Muutoksen taustalla on ihmisen papilloomaviruksen (HPV) aiheuttamien suunielusyöpätapausten merkittävä lisääntyminen, vaikka HPV:hen liittymättömän suunielusyövän esiintyvyys on laskenut. Mikäli taudin lisääntyminen jatkuu edelleen, sen on ennustettu ylittävän HPV:n aiheuttaman kohdunkaulan syövän esiintyvyyden vuonna 2020.

HPV-positiivinen suunielusyöpä eroaa monin tavoin HPV-negatiivisesta suunielusyövästä. Potilaat, jotka sairastuvat taudin HPV-positiiviseen muotoon, tyypillisesti tupakoivat vähemmän ja käyttävät vähemmän alkoholia. Lisäksi emokasvain on yleensä pienempi, mutta kaulalle on usein kehittynyt enemmän etäpesäkkeitä. Se on usein huonosti erilaistunut ja siinä on lukuisia molekyylibiologisia eroavaisuuksia, kuten immunohistokemiallisesti todettava proteiinin p16 vahva ilmentyminen, mikä liittyy HPV:n esiintymiseen kasvaimessa.

Suunielusyöpä hoidetaan usein joko leikkauksen ja sen jälkeisten onkologisten liitännäishoitojen yhdistelmällä tai ns. definitiivisellä onkologisella hoidolla, jolloin leikkaus tehdään vain, jos potilaalla todetaan jäännöskasvain. Samalla kun suunielusyövän esiintyvyys on lisääntynyt, taudin hoitona on käytetty yhä useammin definitiivistä onkologista hoitoa. Suunielusyövän hoitomenetelmät vaihtelevat maittain. Joissakin maissa kansallinen hoitosuositus suosittaa ensisijaiseksi hoitomuodoksi kemosädehoitoa, kun taas toisissa maissa leikkaushoidolla on merkittävämpi osuus.

HPV-positiivisen suunielusyövän lisääntyminen on luonut tarpeen muuttaa hoitosuosituksia, sillä kyseistä tautia sairastavilla potilailla vaste kemosädehoidolle on tyypillisesti parempi kuin virusnegatiivista tautia sairastavilla. Taudin hoidot ovat potilaille raskaita ja ne useimmiten heikentävät elämänlaatua. HPV-positiivista tautia sairastavat potilaat ovat usein nuorempia ja heillä on suurempi todennäköisyys välttyä taudin uusiutumalta, joten osa heistä joutuu kärsimään pitkään hoitojen jälkeisistä haittavaikutuksista. Hoitojen keventäminen HPV-positiivista tautia sairastavien kohdalla saattaa tarjota avun tähän ongelmaan. Meneillään on useita prospektiivisiä satunnaistettuja tutkimuksia, joiden päämääränä on selvittää kevennettyjen hoitojen tehoa suunielusyövässä.

Kliiniseen käyttöön sopivien ennusteellisten biomerkkiaineiden löytäminen voisi mahdollistaa suunielusyöpöpotilaiden yksilöllisempien hoitojen suunnittelun. Tällä hetkellä monen biomerkkiaineen ennusteellista merkitystä tutkitaan tämän syövän suhteen, mutta ainoat validoidut ennusteelliset merkkiaineet ovat HPV ja proteiini p16.

HPV:n aiheuttamassa suunielusyövässä on tulehduksellinen tausta, joten luonnollisella immunitetillä voi olla merkittävä vaikutus taudin kehittymiseen. Tollin kaltaiset reseptorit (TLR) aktivoituvat kohdatessaan kehonulkoisia tai –sisäisiä molekyyliarakenteita, mikä johtaa tulehdusreaktion kehittymiseen. HPV-infektion pitkittyessä kohdunkaulassa ja myös kohdunkaulan syövässä on todettu, että usean TLR:n ilmentyminen on poikkeavaa. Tällä perusteella voidaan olettaa, että TLR:llä voisi olla keskeinen merkitys myös suunielusyövässä.

Tämän tutkimuksen ensimmäisessä osakokonaisuudessa analysoitiin vuosien 2000-2009 aikana annettuja hoitoja ja hoitotuloksia kahdessa potilassarjassa. Ensimmäiseen osatyöhön oli kerätty kaikki Suomen viidessä yliopistosairaalassa hoidetut suunielusyöpöpotilaat. Toisessa osatyössä analysoitiin kaulan hoitoa HYKS:ssä potilailla, joilla oli diagnoosivaiheessa kaulalla etäpesäkkeet. Ensimmäinen osatyö selvitti tiedot suunielusyöpöpotilaiden hoidoista, hoitotuloksista ja ennusteeseen vaikuttavista tekijöistä. Tutkimusajanjakson aikana hoidettiin yhteensä 674 potilasta. Tauti, ja erityisesti sen HPV:hen liittyvä muoto lisääntyi tutkimusajanjakson aikana ja definitiivinen onkologinen hoito toteutettiin yhä useammalle potilaalle. Aikaisempaan kansalliseen suunielusyöpötutkimukseen verrattuna nielun sivuseinämän (nielurisan) taudin ennuste oli pysynyt muuttumattomana, mutta nielun etuseinämän (kielen tyven) taudin ennuste oli hieman parantunut. Ennustetta parantavia tekijöitä olivat naissukupuoli, p16:n vahva positiivisuus, varhaiset T ja N luokat ja intensiteettimuokattu sädehoito.

Kaulametastaaseilla on merkittävä vaikutus ennusteeseen. Tämän vuoksi kaulan hoidolla on keskeinen merkitys pään ja kaulan syöpöpotilaita hoidettaessa. Eloönjäämisen todennäköisyys on alhainen, mikäli suunielusyöpä uusiutuu kaulalla. Osakokonaisuuden toisessa osatyössä tutkimme kaulan hoitoa N+ taudissa. Kauladissektioiden suhteellinen määrä väheni samalla kun definitiivinen kemosädehoito yleistyi. Taudin uusiutumismäärät kaulalla eivät kuitenkaan lisääntyneet. Kaulan alueen uusiutumukset kehittyivät potilaille, joiden kaulan kliininen luokitus oli N2b tai korkeampi ja ne kehittyivät usein emokasvaimen vastakkaiselle puolelle. Tulosten perusteella kaulan molemminpuolinen hoito on perusteltua kaikilla potilailla, joilla todetaan kaulametastaasi.

Tutkimuksen toisessa osakokonaisuudessa (osatyöt III-IV) tutkimme TLR:n merkitystä suunielusyövässä. Analysoimme ensin TLR 2, 3, 4, 5, 7 ja 9 sekä p16 ilmentymisen ja HPV-statuksen 35:sta suunielusyövästä. TLR 5, 7 ja 9 ilmentyminen erosi p16-positiivisten ja -negatiivisten sekä HPV-positiivisten ja -negatiivisten suunielusyöpien välillä. HPV-positiivisissa kasvaimissa TLR 5 ja 9 ilmentyminen oli vähäistä, kun taas TLR 7 ilmentyminen oli voimakasta verrattuna HPV-negatiivisiin kasvaimiin. Suunielusyövän ja suuontelosityövän soluviljelykokeissa todettiin vastaavanlaiset löydökset. Näiden tulosten perusteella selvitimme TLR 5, 7, ja 9 merkitystä laajemmassa 202 potilaan aineistossa verraten näiden proteiinien ilmentymistä potilaisiin ja tautiin liittyviin tekijöihin sekä taudin ennusteeseen. Tulokset vahvistivat aiempia löydöksiä. Sen lisäksi havaitsimme, että voimakas TLR 5 ja matala TLR 7 ilmentyminen olivat yhteydessä korkeaan tautikuolleisuuteen HPV-positiivista tautimuotoa sairastavilla potilailla.

5. INTRODUCTION

The oropharynx (OP) is the middle part of pharynx, located at the level of the oral cavity, and between the nasopharynx and hypopharynx. The OP is typically divided into four walls, of which anterior wall includes the base of tongue and vallecula, the lateral wall includes the palatine tonsils and tonsillar pillars, the superior wall includes the uvula and inferior surface of the soft palate, and the posterior wall includes the mucosa between the nasopharynx and hypopharynx. (1)

Patients with oropharyngeal cancer may initially be asymptomatic, but when symptoms are present, and at advanced stage, these typically include a sore tongue or throat, difficulties or pain in swallowing, ear pain, and a change in the voice. Often, a lump on the neck is the only presenting symptom or sign. (1)

Worldwide malignancies arising from the head and neck are the seventh most common group of malignancies (2). The incidence of head and neck squamous cell carcinomas (HNSCCs) has been slowly declining while a simultaneous decrease has occurred in the most important risk factor, tobacco exposure (3). The incidence of oropharyngeal squamous cell carcinoma (OPSCC) has, however, been rising in many Western countries (3-10). This trend is mainly attributed to the etiological involvement of human papillomavirus (HPV) in OPSCC (5, 11, 12). HPV-associated OPSCC differs from their HPV-negative counterparts in many respects. Patients with HPV association are typically younger, have fewer comorbidities, and have a higher socioeconomic status (11, 13). In addition, their disease is more advanced in terms of staging (UICC and AJCC 7th version) (14) and their tumors have a histologically higher grade of differentiation (15). However, their disease is typically more sensitive to treatment, regardless of treatment modality, and they have better survival (16). As these patients are expected to have a longer post-treatment lifetime than HNSCC patients in general, new treatment strategies are warranted in order to avoid treatment-related morbidity and toxicity without worsening the rates of disease control (17). Although patients with HPV-positive OPSCC have a better outcome, and HPV-negative OPSCC patients may benefit from primary surgery (18, 19), it has been stated that the modification of treatment according to HPV status needs to be avoided outside the context of randomized controlled trials (20-22). However, there is considerable interest in tailoring treatment also according to HPV status, as survival differs between HPV-positive and negative disease. OPSCC patients can also be divided into three groups with distinct survival rates, and the group of patients with the lowest risk of death are possibly suitable for treatment de-intensification (14). Besides HPV status, stratification is based on smoking status or a comorbidity

index, and T and N classes (14, 23). Furthermore, the latest TNM classifications of OPSCC patients divide them into two distinct subgroups according to the status of p16^{INK4A} (p16) (24, 25), a surrogate marker reflecting HPV status (26).

Along with the increasing incidence of HPV-positive OPSCC, the treatment paradigm has changed towards a more oncological approach (27, 28). Some national OPSCC treatment protocols suggest that definitive oncological treatment should be the principal treatment modality for OPSCC, and surgery should be used for salvage purposes only (29, 30). However, some other national protocols recommend both an oncological approach and upfront surgery (20, 22).

Due to the inflammatory background in HPV-positive OPSCC, host immunological responses may be different in this form of disease: enhanced immune surveillance with virus-specific antitumor activity has been shown to occur in HPV-related HNSCC (31, 32). Studies on HPV-induced gynecological cervical cancer may offer information regarding the interplay between immunity and cancer. Persisting HPV infection has been shown to interfere with the expression of toll-like receptors (TLRs), the key activators of innate immunity, (33). In addition, TLR expression and TLR activity display modulations in the cervix during cancerous progression and in cancer (34, 35). The role of TLRs in cancer has been shown to be twofold, because on the one hand they mediate antitumor activity, but on the other hand, they may promote carcinogenesis and tumor progression (36).

This study aimed at evaluating the treatment provided and factors affecting the outcome of OPSCC patients in Finland. Management of the neck was considered in more detail. Furthermore, this study aimed at examining the expression and prognostic role of TLRs in OPSCC.

6. REVIEW OF THE LITERATURE

6.1 EPIDEMIOLOGY AND ETIOLOGY

Head and neck cancer is the seventh most common cancer group in both sexes, with almost 700 000 people affected annually worldwide (2). Squamous cell carcinomas (SCCs) account for more than 90% of head and neck malignancies and their most important risk factors are cigarette smoking and heavy use of alcohol (37). Even though the trend in cigarette smoking has declined over the past decades (38), the incidence of head and neck squamous cell carcinomas (HNSCCs) for most sites has not changed or has only slowly declined (3, 5, 8). However, in the OP, the incidence of SCCs has predominantly risen in many countries worldwide (3-10), with the highest risk for HPV prevalence in Western Europe (39). The evolved epidemiologic profile of OPSCC is attributed to oncogenic high-risk (HR) Human papillomaviruses (HPV): In the US, the incidence of HR-HPV-related OPSCC increased by 0.80% annually from 1974 to 2004, while the incidence of HR-HPV-unrelated OPSCC decreased during the same period (11). Notably, it has been estimated that the incidence of HR-HPV-related OPSCC will surpass that of HR-HPV driven cervical cancer by the year 2020 (5). In Sweden, the rise in the incidence of HR-HPV-associated OPSCC may be stabilizing (40). However, a recent publication suggests a parallel increase for HR-HPV-positive and HR-HPV-negative OPSCC in the United Kingdom (41).

In Finland, detailed information on the incidence of OPSCC is still lacking. However, the incidence of pharyngeal cancer (C01, C09-14) has clearly risen since 1968 among males, while over the same period, the incidence among females has remained stable (42) (Figure 1). The male-to-female ratio for the incidence of pharyngeal cancer was 1.7 between 1968 and 1987, 2.7 between 1988 and 2012, and as high as 4.0 in the year 2014. The patient demographics of OPSCC have altered in the course of the HPV-era: HR-HPV-related OPSCC patients are characteristically younger, and more often male and white than patients with HR-HPV-unrelated OPSCC (11).

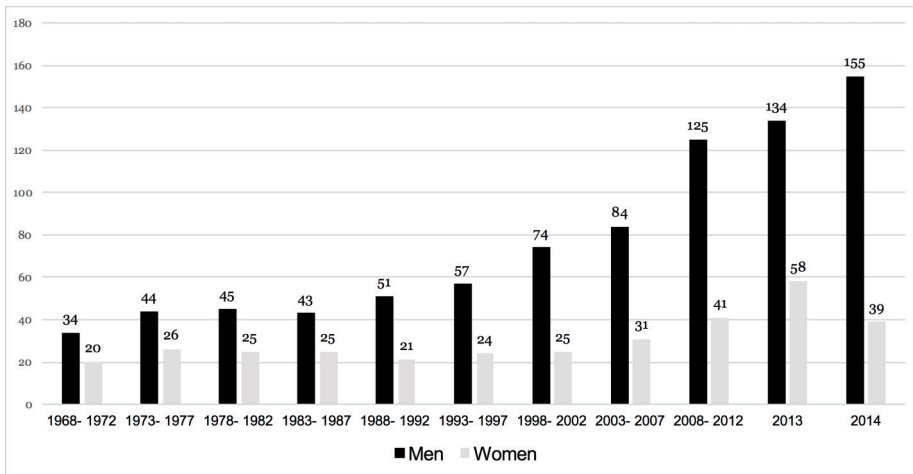


Figure 1. Annual number of new pharyngeal cancer patients (C01, C09-C14) separately for men and women in Finland from 1968 to 2014 (42).

Oropharyngeal HR-HPV infection is most likely responsible for the development of HR-HPV-related OPSCC, as the prevalence of oncogenic HR-HPV is significantly higher in OPSCC patients than in healthy controls (43, 44). Among the male population, the overall incidence of oral HR-HPV infection is reportedly 1.7%, and infections typically clear within one year (45). Evidently, active smoking delays the clearance of oral HR-HPV infection (46, 47). Uncleared oral HR-HPV infections are associated with a risk of developing HR-HPV-related OPSCC (48, 49). The reason why males and whites are at greater risk of HR-HPV infection is probably associated with sexual behavior: a high number of oral sexual partners increases the odds of oral HR-HPV infection (50). The demographic profile of HR-HPV-related OPSCC patients differs from those with a HR-HPV-unrelated OPSCC. Those patients who carry a HR-HPV-positive tumor are typically younger, more probably male, and have a higher socioeconomic status compared with those who have a HR-HPV-negative tumor (11, 13). Recent literature, however, conflicts with the viewpoint that HPV is merely a sexually transmitted disease. It has been shown that sexually inexperienced children also carry HR-HPV-specific cell-mediated immunity (51, 52). Furthermore, the first HR-HPV infection may occur during the fetal period, which may predispose to a prolonged skew in the balance of Th1- and Th2-mediated immune responses, possibly resulting in persistent HR-HPV infection. Another suggested course for HR-HPV persistence is a specific defect in the immune system. (53)

6.2 PATHOGENESIS

HR-HPV-positive OPSCC typically develops in the palatine tonsils or in the base of tongue, whereas the prevalence of HR-HPV in superior wall or

posterior wall tumors is significantly lower (54). Tonsillar tissue has a significant role in the development of OPSCC. The structure of the palatine and lingual tonsils includes a surface layer and crypts, which are histologically distinct. HR-HPV-positive OPSCC typically develops in the crypts, where the epithelium captures and processes antigens, thereby facilitating the entry of HR-HPV into basal cells. HR-HPV-negative OPSCC instead develops in the surface layer of the tonsils (55).

The development of OPSCC largely relies on the abnormal activity of two intracellular cascades, the first involving protein p53, and the second involving proteins p16^{INK4A} (p16), cyclin D1 (cD1), cyclin dependent kinases (CDKs), and retinoblastoma (pRB) (Figure 2).

The p53 protein, first described in 1979, is a transcription factor, which acts as a tumor suppressor. It is activated by cellular stress, such as DNA damage, telomere shortening, oxidative stress, and hypoxia. p53 activity may lead to DNA repair and cause transient growth arrest, but severe cellular stress may also lead to senescence and apoptosis (56).

In the other cascade typically involved in OPSCC, under-phosphorylation of pRB keeps transcription factor E2F unreleased, thereby regulating the G1/S checkpoint. Upstream of these proteins, p16 suppresses and cD1 activates CDKs, which act by phosphorylating pRB (57).

These pathways are perturbed both in HR-HPV-unrelated (58) and HR-HPV-related OPSCC (59), but the underlying mechanisms are different as presented in Figure 2. In addition, HR-HPV markedly affects to the genetic landscape of OPSCC: The rate of mutations in HR-HPV-positive OPSCC is typically half that in HR-HPV-negative counterparts (60). Thus, it has been suggested that HR-HPV-related OPSCC comprises a distinct disease entity (15).

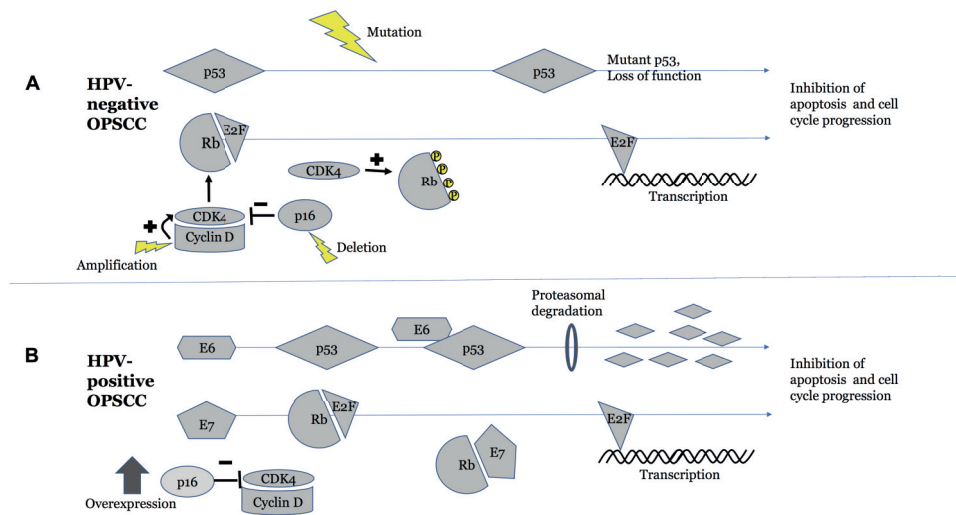


Figure 2. Cancerous pathways in HR-HPV-negative and HR-HPV-positive oropharyngeal squamous cell carcinoma.

A: A mutation in *p53* leads to the loss of its tumor-suppressing function. *Cyclin D1* amplification, and a deletion in *p16* increase the activity of *CDK4*. *CDK4* phosphorylates *pRB*, which loses its repressive function on *E2F*. Inhibition of apoptosis and cell cycle progression results from *p53* inactivity and *E2F* activation.

B: Proteins *E6* and *E7* of HR-HPV are carcinogenic. *E6* binds *p53*, causing *p53* degradation. *E7* binds *pRB* in a phosphorylation-independent manner, causing *E2F* release and activation. Inhibition of apoptosis and cell cycle progression results from *p53* degradation and *E2F* activation.

6.2.1 HR-HPV-UNRELATED OROPHARYNGEAL SQUAMOUS CELL CARCINOMA

Studies have shown that risk factors such as smoking of tobacco (14) and heavy drinking of alcohol (15) are strongly associated with HPV negativity in OPSCC. In addition, poor oral and dental hygiene have been recognized as independent risk factors (61). The key genetic alterations in this form of disease are as follows: a mutation in tumor suppressor gene *TP53* encoding *p53*, a deletion in tumor suppressor gene *CDKN2A/B* encoding *p16*, and amplification in proto-oncogene *CCND1* encoding *cD1* (59). Figure 2A illustrates the pathogenesis of HPV-negative OPSCC. A cell with a *TP53* mutation suffers from a loss of *p53* function, leading to events such as the disruption of DNA repair, growth arrest, and apoptosis (56). A deletion in *CDKN2A/B* leads to the loss of *p16*, ending the negative regulation of CDKs. Amplification in *CCND1* causes increased transcription of *cD1*, and increased activation of

CDKs. This activation of CDKs eventually leads into pRB phosphorylation and E2F activation (59).

6.2.2 HR-HPV-RELATED OROPHARYNGEAL SQUAMOUS CELL CARCINOMA

HPV is an enveloped, non-capsuled double-stranded DNA virus with 210 currently known subtypes (62). The subtypes are further classified into HR and low-risk (LR) subtypes on the basis of their carcinogenic potential (63). In OPSCC, the most important HR-HPV subtype is HPV16, which accounts for nearly 90% of all HR-HPV-positive OPSCC cases, whereas the involvement of HPV18, and other HR-HPV subtypes is rare (64). During HR-HPV infection, the viral genome is integrated into the genome of the host cell, but HR-HPV-DNA can also act intracellularly as an episome. The genome of HPV carries oncogenes, of which E6 and E7 are the most important in the carcinogenesis of HR-HPV-related OPSCC. Both HR-HPVs and LR-HPVs carry these proteins in their genome, but only HR-HPV E6 and E7 have a high affinity for tumor suppressor proteins (65). Oncoprotein E6 binds to p53 and marks it for degradation, thereby preventing its normal function, as presented in Figure 2B. E6 may also cause telomerase activation, leading to immortalization of the infected cell. Oncoprotein E7 binds to and inactivates pRB in a phosphorylation-independent manner, leading to the cessation of its repressive function on E2F. Because of this perturbation, a solution is pursued but is not reached by overexpressing p16 in order to prevent the phosphorylation of pRB. (58) A common mutation occurring in 24% to 31% of HR-HPV-related OPSCCs is a *PIK3CA* mutation causing PIK3 protein activation, the clinical importance of which remains incompletely understood (66-68). In addition, it has been reported that mutations in the gene encoding tumor necrosis factor receptor-associated factor 3 (TRAF3) protein are unique to HR-HPV-associated OPSCC (69).

6.3 DIAGNOSIS

6.3.1 SYMPTOMS AND SIGNS

Oropharyngeal malignancies may be asymptomatic. When present, symptoms may include a sore tongue or throat, difficulties or pain in swallowing, voice change, and ear pain. A lump on the neck is a common initial clinical finding. Other signs include an ulcerated or exophytic mass in the pharynx or an enlarged tonsil. (1)

6.3.2 DIAGNOSTICS

A routine clinical otorhinolaryngological examination is supplemented with endoscopy of the upper aerodigestive tract at an out-patient clinic and under general anesthesia when required in order to evaluate the extent of the disease. The primary tumor is biopsied in the course of an appointment when possible, but particularly in anterior-wall tumors, biopsy under anesthesia may be required. Imaging of the primary site and neck generally includes either magnetic resonance imaging (MRI), or computer tomography. Soft tissue spread is better discerned with MRI, but computer tomography is better in evaluating the degree of bony invasion (70, 71). Diffusion-weighted MRI may enable the discrimination of malignant tissues from non-malignant, and SCCs from other malignancies. In addition, it may be used to detect small cervical lymph node metastasis, distinguish necrotic tissue from viable and post-treatment changes from tumor tissue, and even predict the treatment response. (72) Potential distant spread and second primary tumors are generally detected with computer tomography of the thorax and upper abdomen.

Carcinoma of unknown primary (CUP) presents in 2–3% of HNSCC patients with cervical lymph node metastasis (73). Up to 90% of cases with subsequent primary tumor identification may originate from the OP (74). If fine-needle aspiration cytology (FNAC) from a cervical lymph node reveals signs of SCC with positive p16 staining, the primary tumor is most probably located in the OP (75). According to Danish guidelines (76), which are similar to Finnish practice, if a lump on the neck is the only sign, ultrasound with FNAC is performed. In the case of SCC suspicion in FNAC, the location of the primary tumor is sought using 18F-fluoro-2-deoxy-2-d-glucose positron emission tomography (FDG-PET) computer tomography. FDG-PET computer tomography is followed by tonsillectomy, pharyngo-laryngoscopy, and upper esophagoscopy, and sometimes with other endoscopic procedures. FDG-PET can also be applied with MRI (77). Narrow band imaging can be used to improve the detection of upper airway lesions (78). If a primary tumor is not detected after panendoscopy and tonsillectomy, transoral robotic surgery (TORS) in the identification of base of tongue tumors may be beneficial especially in cases with p16-positive metastases: a study reported that the primary tumor originated in the base of tongue in 78% of such cases (76). If the primary tumor is identified using TORS, the pathologic staging is enabled and the requirement for oncological treatment can be estimated more accurately (76).

6.3.3 HISTOPATHOLOGY

In addition to normal hematoxylin-eosin (H&E) staining of formalin-fixed samples, p16 immunohistochemistry reflecting HR-HPV involvement (26) is also nowadays routinely performed from OPSCCs. The HR-HPV status can be directly detected using the HPV polymerase chain reaction or HPV *in situ* hybridization (ISH), which are discussed in more detail later. Surgical resection samples provide information on tumor size, margins, the depth of invasion, angioinvasion and perineural invasion, and the number and size of lymph node metastases and their extranodal extension (ENE).

6.3.4 STAGING

Clinical TNM staging, based on the primary tumor, regional lymph node metastasis, and distant metastases for OPSCC according to UICC and AJCC 7th edition, is presented in Table 1. The 8th edition of UICC and AJCC TNM staging for OPSCC, published in 2016, separately evaluates the extent of HR-HPV-related and HR-HPV-unrelated OPSCC based on p16 overexpression status (Table 2). The updates to the staging system include new T and N classifications for p16-positive OPSCC, and renewed N classification for all p16-negative HNSCCs. In addition, the clinical and pathologic classification for p16-positive OPSCC are markedly different.

Table 1. TNM and stage classification for oropharyngeal squamous cell carcinoma according to UICC and AJCC 7th edition (79, 80)

Clinical and pathologic T

| | |
|-----|--|
| Tx | Primary tumor cannot be assessed |
| T0 | No evidence of primary tumor |
| Tis | Carcinoma in situ |
| T1 | ≤2 cm |
| T2 | >2 cm but ≤4cm |
| T3 | >4 cm or extension to lingual surface of epiglottis |
| T4a | Invasion to the larynx, deep or extrinsic muscle of the tongue, medial pterygoid, hard palate, or mandible |
| T4b | Invasion to lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, or skull base or encasement of the carotid artery |

Clinical and pathologic N

| | |
|-----|---|
| Nx | Regional lymph nodes cannot be assessed |
| N0 | No regional lymph node metastasis |
| N1 | Metastasis in a single ipsilateral lymph node ≤3 cm |
| N2a | Metastasis in a single ipsilateral lymph node >3 cm but ≤6 cm |
| N2b | Metastasis in multiple ipsilateral lymph nodes ≤6 cm |
| N2c | Metastasis in bilateral or contralateral lymph nodes ≤6 cm |
| N3 | Metastasis in lymph node >6 cm |

M

| | |
|----|-----------------------|
| M0 | No distant metastasis |
| M1 | Distant metastasis |

Clinical and pathologic stage

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

Table 2. TNM and stage classification for p16-positive and p16-negative oropharyngeal squamous cell carcinoma according to UICC and AJCC 8th edition (24, 25)

p16-positive oropharyngeal squamous cell carcinoma

Clinical and pathologic T

| | |
|----|--|
| T0 | No primary identified |
| T1 | ≤2 cm |
| T2 | >2 cm but ≤4 cm |
| T3 | >4 cm or extension to lingual surface of epiglottis |
| T4 | Invasion to the larynx, deep or extrinsic muscle of the tongue, medial pterygoid, hard palate, or mandible or beyond |

Clinical N

| | |
|----|---|
| Nx | Regional lymph nodes cannot be assessed |
| N0 | No regional lymph node metastasis |
| N1 | ≥1 ipsilateral lymph node, <6 cm |
| N2 | Contralateral or bilateral lymph nodes, <6 cm |
| N3 | Lymph node(s) >6 cm |

Pathologic N

| | |
|----|---|
| Nx | Regional lymph nodes cannot be assessed |
| N0 | No regional lymph node metastasis |
| N1 | Metastasis in ≤4 lymph nodes |
| N2 | Metastasis in >4 lymph nodes |

Clinical stage

| | N0 | N1 | N2 | N3 |
|----|-----|-----|-----|-----|
| T0 | NA | I | II | III |
| T1 | I | I | II | III |
| T2 | I | I | II | III |
| T3 | II | II | II | III |
| T4 | III | III | III | III |

Pathologic stage

| | N0 | N1 | N2 |
|----|----|----|-----|
| T0 | NA | I | II |
| T1 | I | I | II |
| T2 | I | I | II |
| T3 | II | II | III |
| T4 | II | II | III |

Any M1 is stage IV

p16-negative oropharyngeal squamous cell carcinoma

Clinical and pathologic T

T classification: similar to UICC and AJCC 7th edition (class T0 no longer included)

Clinical and pathologic N

| | |
|----|--|
| Nx | Regional lymph nodes cannot be assessed |
| N0 | No regional lymph node metastasis |
| N1 | Metastasis in a single ipsilateral lymph node, ≤3cm and no ENE |

| | |
|-----|--|
| N2a | Metastasis in a single ipsilateral lymph node >3 cm but ≤6 cm and no ENE |
| N2b | Metastasis in multiple ipsilateral lymph nodes, <6 cm and no ENE |
| N2c | Metastasis in bilateral or contralateral lymph nodes, <6 cm and no ENE |
| N3a | Metastasis in a lymph node >6 cm and no ENE |
| N3b | Metastasis in any node(s) and clinically overt ENE |

Clinical and pathologic stage

| | | | | |
|-----|-----|-----|-----------|--------|
| T | N0 | N1 | N2a, b, c | N3a, b |
| T1 | I | III | IVA | IVB |
| T2 | II | III | IVA | IVB |
| T3 | III | III | IVA | IVB |
| T4a | IVA | IVA | IVA | IVB |
| T4b | IVB | IVB | IVB | IVB |

Any M1 is stage IVC

ENE = Extranodal extension

6.4 TREATMENT

Early-stage OPSCC can usually be managed with single-modality treatment. Advanced-stage disease requires multimodality treatment, which has typically consisted of a combination of surgery and postoperative radiotherapy (RT) or CRT. The treatment modality for the neck is usually the same as that for the primary site. However, definitive oncological treatment, also known as organ-sparing treatment, currently has a significant role in this setting. This method is complemented by salvage surgery in cases of residual or recurrent disease. (1)

6.4.1 TREATMENT OF THE NECK

A decisive step in neck management is to evaluate whether the neck is clinically classified as No (cNo) or N+ (cN+). Elective neck treatment is typically delivered if the risk of neck metastasis exceeds 20%, and it can consist of either an elective neck dissection (ND), or elective RT. (81) Most OPSCC patients are diagnosed with cN+ status requiring therapeutic treatment (82). Furthermore, even in most OPSCC patients with cNo status, the estimated risk of metastasis exceeds a level requiring neck treatment (82). An exception to this are e.g. patients with T1No soft palate tumors with a lower risk of metastasis and in whom a sentinel lymph node biopsy (SNLB) may also be an option to evaluate the neck status more accurately (83, 84).

6.4.2 GENERAL ASPECTS OF SURGERY

6.4.2.1 SURGERY OF THE PRIMARY SITE

Surgery is an option for the majority of oropharyngeal tumors, but indications for resection largely depend on factors such as the tumor site, and the expected impact of treatment on patient function and quality of life. A 1–2 cm margin of healthy tissue has to be taken into the resection specimen with an aim to achieve a minimum of 5 mm microscopical margins, which in advanced tumors leads to large defects. Reconstruction for a large defect is typically performed with a microvascular free tissue transfer, such as an anterolateral thigh flap or a radial forearm flap. Most small tumors can be operated transorally, and larger ones require wider open exposures through the neck, and sometimes mandibulectomy. (1) TORS is an alternative, especially in the resection of small or moderate-sized base of tongue tumors (85).

6.4.2.2 SURGERY OF THE NECK

The extent of ND depends on the primary tumor site and T class, as well as N class. However, the exact levels that should be included in the ND are to some extent debatable (1, 86). Elective ND, which generally includes levels I–IV in OPSCC, also provides valuable pathologic information regarding regional disease, which may influence the treatment intensity (81). In cN+ disease, levels I–V should generally be dissected (1). The risk of contralateral nodal involvement is increased if T class is T4 or N class is N2a or higher (82). Lim et al. have recommended that if tonsillar SCC is classified as T3 or higher, and the patient presents with ipsilateral metastasis, contralateral ND should also be performed (87).

6.4.2.3 SALVAGE SURGERY

Treatment response and the need for surgical salvage for a residual tumor after definitive oncological treatment are currently evaluated with PET-CT at 3–4 months after treatment completion, as the rate of false-positive and possible false-negative findings may be higher if PET-CT is performed earlier (88). A negative PET-CT is, however, fairly reliable, with a negative predictive value around 95% (89). If PET-CT shows activity at the primary site, biopsies should be taken to confirm or exclude residual disease. A positive finding on the neck in PET-CT is an indication for ND. (89, 90) A proportion of post-treatment PET-CT scans are classified as equivocal. In these cases, qualitative interpretation using a Likert scale may enable the possibility of residual disease to be ruled out (91). It has also been suggested that imaging findings could determine the extent of salvage NDs, which would then in most cases include levels II–IV, whereas dissection of levels I and V should be based on pathological findings in images (92, 93). The extent of salvage surgery of the residual or recurrent tumor is planned according to the dimensions of the primary tumor before CRT, often requiring large resections (94, 95). In oropharyngeal tumors, however, the radicality of the salvage resection may sometimes be limited because of the close proximity of vital structures such as the internal carotid artery and skull base, and this possibly accounts for inferior cure rates reported for local residual OPSCC compared with local residual hypopharyngeal SCC (94).

The rate of complications is particularly high after salvage surgery (94, 96), and patients have a high risk of a significant loss of function (97). Salvage surgery for recurrences does not particularly differ from salvage surgery performed for residual tumors, and survival in both patient populations remains poor (94).

6.4.3 GENERAL ASPECTS OF ONCOLOGICAL TREATMENT

6.4.3.1 RADIOTHERAPY

Ionizing radiation damages DNA and causes cell death in all tissues, which is why RT should be extremely tumor selective. In addition, the supply of oxygen has a considerable impact on RT efficacy, and hypoxia may reduce the effectiveness of RT (98), but also increase the likelihood of invasion and metastasis due to increased angiogenesis (99).

The delivery of RT for head and neck tumors is complicated because of the close proximity of surrounding organs at risk (OAR), such as major salivary glands, the spinal cord, brainstem, optic nerve, pharyngeal muscles, and vocal cords. In the early 1990s, the 3D conformal RT (3D-CRT) method was introduced. This technique utilizes computer tomography images in dose planning, but the radiation intensity is typically the same across the field or modified with wedges or compensating filters. The shape of the beam is determined using either customized blocks or multiple leaf collimators (MLC). In IMRT, introduced during the late 1990s, computer-assisted determination of the radiation dose to the tumor is applied utilizing multiple radiation beam directions, beam intensity modulation with dynamic MLCs, and other devices. This method enables the delivery of a higher radiation dose to the tumor with lower dose to OAR compared with the 3D-CRT. IMRT, however, requires more labor in the planning phase and is more expensive. (100) Proton therapy, which is not yet available in Finland, may offer advantages in head and neck cancer treatment, as the greatest part of the energy is released at a defined depth. When proton therapy is delivered from different beam angles, the method is termed intensity-modulated proton therapy (IMPT). (101)

RT is given in daily fractions. The fractionation of RT may be conventional, accelerated, or hyperfractionated. In conventional fractionation, a daily dose of 2 gray (Gy) is delivered for five days followed by a break of two days. Generally, the conventional RT takes six to seven weeks to complete. In the accelerated form, the intervals between RT are shortened without a significant change in the single dose or total dose. In hyperfractionated RT, single doses are reduced and the intervals between treatment sessions are shorter without a significant difference in the total treatment time or total dose. (102) In Finland, conventional fractionation is most commonly used.

In definitive oncological treatment, depending on tumor-related factors such as the tumor stage, patients receive a total dose between 66 and 70 gray (Gy). The involved regional lymph nodes and high-risk areas are treated with a total dose of 60-70 Gy. The elective dose is 50 Gy. (100)

After primary surgery, most patients require postoperative oncological treatment. The postoperative RT dose to high-risk areas is typically 60–66 Gy. For intermediate-risk areas, this dose is approximately 60 Gy. Elective areas are treated with a total dose of 50 Gy. (100)

6.4.3.2 CHEMOTHERAPY

In relation to RT, CT may be neoadjuvant, concomitant, or adjuvant. In neoadjuvant treatment, CT is administered before the main treatment in order to reduce the tumor volume to facilitate more effective treatment (103). Neoadjuvant CT is, however, rarely used in OPSCC treatment in Finland. The benefit of concomitant CRT has been shown to be higher than neoadjuvant CT (104). In Finland, the most commonly used CT agent in concomitant CRT is weekly Cisplatin 40 mg/m² (Guidelines of the Finnish Head and Neck Oncology Working Group). Cisplatin 100 mg/m² every third week, carboplatin, 5-fluorouracil, and paclitaxel are seldom-used CT agents. A biological agent, such as cetuximab, may also be used. (100)

6.4.3.3 IMMUNO-ONCOLOGY

Immune-based treatment for HNSCCs has been introduced during recent years, as understanding of the immune system's role in the development of cancer has been increasing. Immunotherapy strategies for HR-HPV-positive and HR-HPV-negative are suggested to be different (105). In HR-HPV-positive OPSCC, viral oncoproteins E6 and E7 present as optional targets for treatment, as they are expressed in the tumor cells, and their suppression may restore the activity of suppressed p53 and pRB (106). Currently, therapeutic vaccination is the most examined immune-based treatment method for HR-HPV-positive OPSCC. Therapeutic vaccines may contain DNA or peptides. In addition, the patient's own dendritic cells can be matured and activated (dendritic cell vaccine) *ex-vivo* to enhance anti-tumor immunity, or the patient's own tumor-specific T-cells can be cultured *ex vivo* and thereafter transferred back into the patient (adoptive T cell transfer). (105) In the treatment of patients with HR-HPV-negative OPSCC, the use of monoclonal antibodies has been studied. In addition, immune checkpoint inhibitors, such as anti-CTLA-4, anti-PD-1 and anti-PD-L1 antibodies are currently being investigated, as well as dendritic cell vaccine and adoptive T cell transfer. (105) Recently, it has been shown that treatment of recurrent HNSCC using anti-PD-1 antibody therapy results in longer survival than after treatment with standard therapy (107). In addition, it has been shown that treatment of recurrent HNSCC with another anti-PD-1 antibody, pembrolizumab, is well tolerated and may result in feasible treatment response (108, 109).

6.4.4 TREATMENT GUIDELINES FOR OPSCC

In Finland, the multidisciplinary National Head and Neck Oncology Working Group maintains and updates national treatment guidelines for HNSCC. The treatment of OPSCC with curative intent in Finland may follow the both of the two main patterns: primary surgical treatment with or without postoperative oncological treatment or, alternatively, definitive oncological treatment with salvage surgery when needed. The treatment decision is recommended individually by a multidisciplinary tumor board, and it depends on both patient-related factors, such as comorbidities, performance status, compliance, and the patient's own wish regarding treatment, and on tumor-related factors such as the extent of disease (TNM) and site. In addition, institutional resources and experience also have an impact in the treatment decision. (Guidelines of the Finnish Head and Neck Oncology Working Group).

Generally, small tumors, such as T1-T2 tonsillar SCCs, may be treated with upfront surgery followed by adjuvant treatment rather than with definitive oncological treatment in order to avoid long-term adverse sequelae such as xerostomia. However, patients with larger tumors, such as T4 base of tongue SCCs, would most probably have a markedly reduced quality of life when treated with upfront surgery compared with definitive oncological treatment. (110) In superior-wall tumors, if more than half of the soft palate has to be reconstructed after resection, the speech outcome is considerably worse (111). Zelefsky et al. (112) reported that quality of life related functional loss after upfront surgery is significantly higher if the primary tumor is classified as T3-T4 and if the tumor originates from the base of the tongue. In their surgically treated series, the List (113) performance rates for eating in public, understandability of speech, and normality of diet were lower (112) than in another series treated with definitive oncological therapy for base of tongue SCC (114). However, a recent systematic review evaluating the swallowing outcome after a trans-oral surgical approach and after definitive oncological treatment concluded that according to the current evidence, direct head-to-head comparisons of the swallowing outcome between these two modalities cannot be made (115). An ongoing study, "Phase II Randomized Trial for Early-stage Squamous Cell Carcinoma of the Oropharynx: Radiotherapy vs Trans-oral Robotic Surgery" (116), may provide valuable information regarding the differences in the functional outcomes after these treatment modalities.

It has to be taken into account that treatment guidelines for OPSCC vary between countries. In Sweden, definitive oncological treatment with salvage surgery when necessary is considered as the principal treatment method, but primary surgery can be considered for T1 superior wall tumors, T1-T2 tonsil and base of tongue tumors (29). In Denmark, the DAHANCA guidelines suggest only a definitive oncological approach, and surgery is preserved as a

salvage modality (30). In the United Kingdom and the US, primary surgery or radiotherapy alone can be considered for early-stage (I-II) OPSCC. Advanced-stage (III-IVb) OPSCC may include either combination treatment, i.e. surgery and postoperative oncological treatment, or definitive oncological treatment with salvage surgery if necessary. (20, 22)

The variation in treatment probably reflects the lack of consensus regarding the optimal treatment. OPSCC etiology has been rapidly changing over the two last decades, and evidence for the optimal treatment is thus lacking. Parallel comparisons of survival outcomes after primary surgical treatment and definitive oncological treatment in randomized-controlled prospective studies are lacking, but separate studies have shown similar outcome figures after these two treatment modalities (14, 110, 117-119). However, the rates of severe and fatal complications after upfront surgery are strikingly higher in tonsillar SCC, and especially in base of tongue SCC, when compared with definitive oncological treatment (110). HR-HPV status is the factor affecting to the survival at the most, but many authors have recommended, at least so far, that modifying the treatment according to HR-HPV status in clinical practice should be avoided, as sufficient evidence for how the treatment should differ is still lacking (20-22). However, there is a growing interest in tailoring treatment according to HR-HPV status, as it has been reported that patients carrying HR-HPV-negative OPSCC may benefit more from primary surgery than definitive oncological treatment (18, 19), and some patients carrying HR-HPV-positive tumors may be suitable for treatment de-escalation (14). It is also noteworthy that the latest TNM classification is different for p16-positive and p16-negative OPSCC (24, 25).

6.4.5 BENEFITS AND DISADVANTAGES OF DIFFERENT TREATMENT MODALITIES

Definitive oncological treatment and surgery combined with oncological treatment have both been alternately considered as the optimal treatment modalities for OPSCC from the early 20th century, and the paradigm shifts have often been attributed to advances in technology (120). From the late 1990s onwards at many institutions, upfront surgery was considered a secondary option, as developments in RT and CT improved survival and reduced treatment-related morbidity. According to a national cancer database in the US, during the years 1985–2001, surgery was performed for 27–28% of all OPSCC patients, while the use of definitive CRT had increased from 15% to 29% (27). In Finland, during 1995–1999, primary surgery was performed for 85.4% of OPSCC patients, and definitive oncological treatment was given to only 14.6% of the patients (121). From 1998 to 2009, OPSCC treatment changed in the US, as the surgical treatment declined from 41% to 31%, and the use of definitive CRT increased from 22.4% to 61.5% (28). Another report

showed that a 2.4-fold decrease in primary surgical treatment and a 4.5-fold increase in CRT occurred in the US between the periods of 1955–1994 and 1995–2004 (122). Definitive oncological treatment became described as an organ-preserving therapy, and it was introduced to offer an advantage in functional outcome, as the tumor sites remain without surgical intervention for those who do not develop residual or recurrent disease (123). In OPSCC, a two-year rate of 84% in organ preservation after definitive CRT has been reported (124). However, a minimally invasive approach in upfront surgery is currently increasing, and a resection can be carried out using TORS. This method aims at reducing the morbidity related to traditional open approaches without worsening survival. Offering surgery as a treatment option also carries an advantage in terms of obtaining a pathological analysis of the disease. As the extent of disease is revealed, adjuvant treatment planning can be considered after surgery, based on the marginal status, pathological T and N staging, and ENE. In addition, after upfront surgery, the dose of RT may be lower and CT can be avoided in some cases. (125)

After definitive CRT, using older 2D- and 3D-RT techniques, the risk of various severe late toxicities may be as high as 12.6% for feeding tube dependence, and 39.6% for laryngeal or pharyngeal dysfunction (126). Importantly, there is evidence that salivary gland function can be well preserved after IMRT without jeopardizing disease-control rates (127, 128). In addition, in OPSCC, IMRT compared with 3D-CRT offers better long-term results in functional outcome, such as swallowing, with no difference in survival (129). Furthermore, in the treatment of OPSCC, IMPT has been shown to reduce feeding tube dependency and severe weight loss without jeopardizing survival (130).

Re-irradiation of head and neck cancer has traditionally resulted in a markedly high rate of late toxicities such as trismus, osteoradionecrosis, mucosal necrosis, and ultimately even carotid hemorrhage (131). However, delivering IMRT-based re-irradiation has provided a less toxic approach to pursue a cure for head and neck cancer (132). In addition, boron neutron capture therapy (BNCT) has been studied in the treatment of inoperable and irradiated locally recurrent head and neck cancer (133).

CT is delivered to radio-sensitize the tumor, but also to reduce the risk of distant failure. CT agents have however, several adverse effects. The most commonly used agent, cisplatin typically causes nausea, but also toxicity in the nervous system, kidneys, inner ear (134), and bone marrow (135). Another common CT agent, cetuximab, typically causes rash, mucositis, nutrition impairment, hypomagnesemia, and infusion reactions (134).

6.4.6 DISEASE-STABILIZING TREATMENT AND PALLIATIVE TREATMENT

Non-curative treatment always aims at palliating the patient's symptoms. In selected cases, slowing down the progression of the disease may also be pursued. Treatment approaches may include methods also used in curative treatment, such as RT, CT, or surgery (136).

6.5 PROGNOSIS AND PROGNOSTIC FACTORS

6.5.1 GENERAL ASPECTS ON PROGNOSIS

Survival has markedly improved among HNSCC patients since the 1980s, with biggest improvement in tonsillar carcinomas. The reasons underlying this phenomenon may include the involvement of HR-HPV infection and more sophisticated treatment methods, such as IMRT. (137) In addition, as shown in the latest update of a meta-analysis, concomitant CT as a part of the treatment for HNSCC reduces the 5-year mortality by up to 6.5% (104).

6.5.2 PROGNOSTIC FACTORS

6.5.2.1 *T and N class and stage*

T and N class have been regarded as the most important factors affecting the outcome of OPSCC (138-140). Stage, a derivative of T and N class reflecting the extent of disease, has also been shown to independently impact on the outcome (141, 142). More recently, survival analysis adjusted for HR-HPV status has suggested that in HR-HPV-negative OPSCC, stage (143) and N class (144), but not so clearly T class (143, 144), influence survival. Interestingly, in HR-HPV-positive OPSCC, only T class seems to be prognostic (143, 144). It is, however, well established that the UICC and AJCC 7th editions of the TNM staging (79, 80), which are not based on HR-HPV association, do not reflect patient survival as well as before the HPV-associated era (122).

6.5.2.2 *HR-HPV status and p16 overexpression*

It is widely established that patients with HR-HPV involvement have better survival in OPSCC (145, 146), and that HR-HPV status is currently the best marker in the prognostication of OPSCC (14, 23). A meta-analysis has shown

that patients with HR-HPV-positive OPSCC have 28% lower five-year overall mortality, and a 49% higher disease-free survival rate (146). The better treatment response among HR-HPV-positive tumors is suggested to be due to better radiosensitivity (117, 147, 148). The treatment outcome of HR-HPV-positive OPSCC, however, seems to be modality independent (16). Better outcome among HR-HPV-positive tumors may be associated with the absence of viral field cancerization, reducing the risk of locoregional recurrence and secondary primary tumors (149), and with enhanced immune surveillance and virus specific antitumor activity (31, 32). However, an aggressive neuroendocrine variant of HR-HPV-positive OPSCC with positivity for synaptophysin, chromogranin, and CD56 has also been recognized (69).

HR-HPV status determination has some shortcomings in sensitivity, specificity, and accessibility. Tests that are based on the HR-HPV-DNA polymerase chain reaction (PCR) may be oversensitive and lack sufficient specificity. Therefore, the positivity may also indicate a non-transcriptional HR-HPV infection (150). Tests that are based on HR-HPV-DNA ISH may have insufficient sensitivity, while their specificity is good. ISH results are analyzed in the histological context, and if staining is only positive in tumor cells, the positivity can be regarded as a sign of transcriptional HR-HPV infection (151). HPV E6/E7-mRNA PCR may only detect transcriptionally active, relevant, HR-HPV infection, and it is therefore considered as the most reliable test for HR-HPV involvement (152). However, it usually requires fresh frozen tissue specimens. Protein p16 overexpression due to E7 binding on pRB can be detected with immunohistochemistry, a method available in routine laboratories. p16 overexpression (>70% of the tumor tissue stains strongly) can be used as a surrogate marker for HR-HPV with good sensitivity, but moderate specificity (153). Protocols that reliably identify tumor HR-HPV status, combine the strengths of p16 immunohistochemistry (IHC) and HR-HPV detection. According to the method suggested by Smeets et al. (152), p16 IHC is performed on all samples and p16 negatives are considered as HR-HPV negative. HR-HPV PCR is performed for the p16-positive samples, and only the samples that are positive for both p16 IHC and HR-HPV PCR are classified as HR-HPV positive. Furthermore, a combination of p16 IHC and HR-HPV ISH can be performed in a similar manner to improve the classification of samples as HR-HPV positive (154).

HR-HPV status may have an interaction with certain factors, such as T and N classification and stage, and thereby modify both their odds and prognostic significance (143, 144). However, the new AJCC 8 staging for OPSCC, which is based on the p16 status of the tumor, supposedly improves the prognostication of TNM (155).

6.5.2.3 Smoking and heavy drinking

Smoking (14) and heavy use of alcohol (15) may affect the outcome of OPSCC independently of HR-HPV status. One pack-year of smoking accounts for a 1% increase in the annual mortality risk (14). Similarly, heavy alcohol consumption causes a 2.6-fold increase in the mortality risk (15). The interaction between smoking and alcohol in the risk of developing pharyngeal cancer is well known, (156), but their potential synergistic effect on survival remains open.

6.5.2.4 Other prognostic biomarkers

For further classification of the prognosis in OPSCC, complementary markers to HR-HPV are required. Previously, an association has been suggested between a poor outcome and high expression of markers including HIF- α (157), c-met, Bcl-2 (158), Ki-67 (159), a combination of VEGF and EGFR (160), Cox-2 (161), nuclear survivin (162), MVP (163), phosphorylated serine/threonine protein kinase B (AKT) (164), heregulin mRNA, HER 3 (165), cytoplasmic Bmi-1 (166), and FHIT (167). In addition, loss of the 16q gene and the absence of 11q13 gene amplification have been shown to be associated with a good outcome (168). It is, however, noteworthy that validation of these suggested markers remains fairly limited in OPSCC research (169).

6.6 TREATMENT DE-ESCALATION

In 2010 Ang et al. reported that OPSCC patients can be divided into three different groups with distinct outcomes according to HR-HPV status, smoking pack years, and T and N classes (14). Based on their results, they suggested that de-intensification of treatment for the HR-HPV-positive group with the best outcome is justified. As patients with HR-HPV-positive OPSCC are generally younger and have a higher probability of a more favorable treatment response and better survival, treatment de-intensification in order to minimize post-treatment adverse effects without increasing the risk of failures seems to be well justified (170). OPSCC treatment generally requires multimodality treatment with high doses of radiation, and these patients may experience several late toxicities such as xerostomia, swallowing difficulties and pain, and ototoxicity. New treatment strategies with treatment de-escalation are therefore warranted (171). Several de-escalation studies are ongoing with an aim to evaluate the benefit of cetuximab instead of cisplatin, de-intensification of RT doses, induction CT before de-intensified RT doses, and less invasive surgery (170).

The locoregional control rate in HR-HPV-positive OPSCC is typically good, and the failures seem to predominately occur at distant sites (172). O’Sullivan et al. demonstrated that patients with HR-HPV-positive OPSCC and N2c classification treated with RT alone had a distant control rate of only 73%, while for those who received CRT, the corresponding figure was 92% (173). Therefore, they suggested that de-intensification from CT should be avoided in patients who are considered to be at high risk of developing distant metastasis (173). Most de-escalation studies use p16 overexpression in HPV status determination due to practical reasons, which has also been criticized (170). Patients who have p16-positive, but HPV-negative OPSCC tend to have survival rates resembling those of patients who have p16-negative and HR-HPV-negative OPSCC (23). In fact, the tumors of the former group have genetically more similarities with HR-HPV-negative tumors than with HR-HPV-positive tumors (174).

6.7 PREVENTION OF HR-HPV-ASSOCIATED OPSCC

A total of 5% of all cancers worldwide are attributed to HPVs (175). The first prophylactic HR-HPV vaccine was approved for the prevention of cervical carcinoma in 2006 (176). Subsequently, vaccines preventing infections of more HPV types have been introduced, and vaccination has also been studied in the prevention of cancers arising from other sites than the cervix (176). Vaccines have been shown to be highly effective against HR-HPV and sequel anogenital precancerous lesions (177). As the most important HR-HPV types responsible for OPSCC are HPV-16 and HPV-18, it has been debated whether vaccine prevention of HR-HPV-associated OPSCC would be relevant (64). In the oropharynx, the detection of precancerous lesions using cytology has been shown to be unfeasible, possibly because the relevant tonsillar epithelium is difficult to sample (178). However, although the vaccination effect on precancerous lesions may be difficult to prove, vaccination has been shown to significantly reduce the prevalence of oral HR-HPV infections, possibly implying that vaccination may have a role in the prevention of OPSCC (179).

6.8 TOLL-LIKE RECEPTORS

Innate immunity is in a key position in the initiation of the inflammatory response in a process mediated by toll-like receptors (TLRs), which are normally expressed by various immune cells and epithelial cells located near the host–environment boundary (180, 181). These receptors are activated by molecular patterns, such as proteins, RNA, or DNA strands, which can be either exogenous or endogenous. Exogenous patterns are called pathogen-associated molecular patterns (PAMPs) (180, 181) and endogenous ones are

called danger-associated molecular patterns (DAMPs) (182, 183). TLRs belong into a family of 10 transmembrane receptor proteins, of which TLR 1, 2, 5, 6, and 10 have an extracellular receptor domain, and TLR 3, 4, 7, 8, and 9 have their receptor domain located inside an intracellular vesicle (184). Downstream of TLRs there are multiple cascades, the most important of which results in the activation of nuclear factor κ B (NF- κ B), which controls the transcription of proinflammatory cytokines, but also proteins participating in cell survival and proliferation (Figure 3). TLRs can also activate mitogen-activated protein kinase and TRAF-3 mediated pathways (184). Under normal conditions, TLR activation maintains systemic homeostasis by defending the organism by triggering immunity towards harmful molecular structures. However, TLR activation may also result in inflammation becoming chronic, which may offer a favorable basis for cancer development (36).

6.8.1 TOLL-LIKE RECEPTORS AND CANCER

The first observations indicating an association with TLRs and cancer were made without modern knowledge of immunology. In the 1890s, Dr. Coley showed that the administration of killed bacterial extracts resulted in antitumor activity (185). Another finding that nowadays is considered to be related to TLRs occurred earlier in the 1900th century, when Dr. Virchow noticed that cancer often developed into a site with chronic inflammation (186). Today, an association is known to exist between chronic inflammation and various types of cancer, such as *Helicobacter pylori* infection with gastric cancer (187), HPV with cervical cancer (188), ulcerative colitis and Crohn's disease with colon cancer (189), and hepatitis infections with hepatocellular cancer (190).

Many studies have shown that TLRs are overexpressed in premalignant and cancerous cells or tissues (191-194), indicating that TLRs play an important role in carcinogenesis and tumor progression. TLRs can modulate the immunity of the tumor microenvironment, increasing the levels of immunosuppressive agents such as vascular endothelial growth factor and transforming growth factor beta, and therefore increase angiogenesis and the odds of metastasis (195). In addition, TLRs can activate immunity by secreting nitric oxide synthase 2 and cyclooxygenase 2, which may cause tumor progression (191, 196). Activation of TLRs can, however, also lead to the opposite result: tumor inhibition by activating the immunologic response towards cancer (197-200).

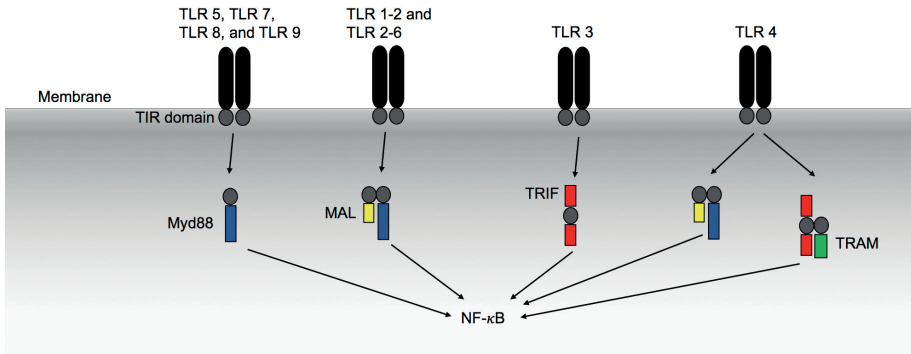


Figure 3. Activation of *Nf-κB* following TLR activation. Intracellular *Myd88* is recruited to activate *Nf-κB*. In the case of TLR 1-2, TLR 2-6, and TLR 4 activation, bridging adapter MAL is recruited. TLR 3 activation is followed in a *Myd88* independent manner using TRIF as an adapter. TLR 4 activation may be followed by the *Myd88* pathway where MAL is involved or the *Myd88*-independent pathway with adapter proteins TRIF and TRAM. (Modified and reprinted with the permission of Nature Publishing Group. (201)).

6.8.2 TOLL-LIKE RECEPTORS AS PROGNOSTIC MARKERS AND THERAPEUTIC TARGETS

As the association of TLR expression and cancer development and progression has been well established, numerous cohort studies have shown the relationship between TLR expression and survival in various types of cancer (202-212). In addition, abundant studies on TLR signaling and its consequences in cancers have been published (213). The effect of TLR-mediated cancer treatment can be caused by various processes. It may lead to apoptosis of tumor cells, increased vascular permeability and thus tumor regression, but also directly or indirectly recruit leukocytes and thus activate natural killer cells and T cells (214). The pursued anticancer activity of TLR agonists is thought to be a consequence of the activity of myeloid cells such as monocytes, macrophages, and dendritic cells (215). However, TLRs are also expressed on cancer cells, and many studies have pointed out their role in cancer progression or regression (213). Numerous clinical trials on TLR agonists are ongoing (215), and TLR agonists are currently used in the treatment of urinary bladder carcinoma (215, 216) and basal cell carcinoma of the skin (215, 217).

7. AIMS OF THE STUDY

The first part of this study analyzed the treatment and outcome of OPSCC in two patient series, the first comprising all patients treated at the five Finnish university hospitals and the second focusing on the management of the neck in cN+ disease in patients treated at Helsinki University Hospital. The second part of this study examined the role of TLRs in OPSCC.

The specific aims of this study were:

1. To gather information on the given treatment, outcome, and prognostic factors in a multicenter cohort including all patients diagnosed and treated at the five Finnish university hospitals between 2000 and 2009. Special emphasis was given to the change in the treatment approach;
2. To evaluate the management and outcome of the cN+ neck in OPSCC;
3. To examine the expression of TLR 2, 3, 4, 5, 7, and 9 in HR-HPV-positive and HR-HPV-negative OPSCC *in vivo* and *in vitro*;
4. To study the prognostic influence of TLR 5, 7, and 9, and their association with clinical parameters in OPSCC in a patient series.

8. MATERIALS AND METHODS

8.1 PATIENTS AND STUDY DESIGN

8.1.1 Study I

Study I covered all patients who were diagnosed and treated for OPSCC at the five Finnish university hospitals between January 1st, 2000 and December 31st, 2009. A total of 674 patients were identified, and 600 of them received treatment with curative intent.

At each university hospital, information was manually collected from hospital patient records regarding clinicopathological data, treatment, and outcome. Formalin-fixed paraffin blocks were collected from the archives of the Department of Pathology at each university hospital, and p16 IHC on formalin-fixed paraffin blocks was carried out retrospectively. The p16 status of the primary tumors was determined for the patients treated with curative intent, and of these, tumor tissue was available in 431 (71.8%) cases. The dates and causes of death were provided by Statistics Finland. Patients with subsequent follow-up, or treatment carried out at a central hospital according to the recommendation of the university hospital multidisciplinary tumor board were included. Treatment was classified as completed if the radiation dose in combination treatment (surgery + RT or CRT) was at least 45 Gy, and in definitive oncological treatment (RT or CRT) at least 60 Gy. If at least one cycle of CT was given, patients were considered to have received CRT. Of the patients who were treated with an intent to cure, 99% and 75% had a minimum follow-up of 3 and 5 years, respectively, or until death.

8.1.2 Study II

Study II included all 313 patients diagnosed and treated for OPSCC at Helsinki University Hospital between January 1st, 2000 and December 31st, 2009. These 313 consecutive patients also represented the Helsinki University Hospital material in Study I. The study focused on 201 patients with regional metastasis (cN+) who were treated with curative intent. Of these, we further analyzed a subgroup of 169 patients who underwent treatment that was considered completed on the same grounds as in Study I, and who had a curative response (CR) for treatment and were disease free at three months after treatment completion. Combination treatment (surgery ± RT or CRT) was delivered to

107 patients and 62 patients received definitive oncological treatment (CRT or RT ± surgery).

8.1.3 Study III

Study III included a cohort (n=35) of OPSCC patients diagnosed at Helsinki University Hospital, as well as five OPSCC cell lines (UT-SCC 65, -69, -60A, -60B, and -102) and one oral tongue cancer cell line (HSC-3). We aimed to evaluate TLR 2, 3, 4, 5, 7, and 9 expression, p16 status, and HR-HPV status *in vivo* and TLR 5 and 7 expression, p16 status, and HR-HPV status *in vitro*.

8.1.4 Study IV

The population of study IV consisted of 331 patients, of whom 18 were excluded due to histology other than SCC or a subtype of SCC. The remaining 313 OPSCC patients were the same patients as in Study II. Furthermore, patients were excluded with palliative intention of treatment (n = 44), concurrent HNSCC (n = 5), earlier-treated HNSCC (n = 11), and unavailability of tumor tissue (n = 51). Finally, a total of 202 patients were analyzed in this study. TLR 5, 7, and 9 expression and p16 status were immunohistochemically determined using the tissue microarray (TMA) method. HR-HPV status was determined using ISH.

8.1.5 Tissue microarray and immunohistochemistry

For TMA blocks, we selected three different areas based on the invasion depth from slides stained with H&E. Six representative 1-mm core biopsies from marked areas of each tumor were placed in a paraffin block with a manual tissue microarrayer (Beecher Instruments, Silver Spring, MD, USA). Two similar blocks were produced from all samples.

Four-micrometer-thick tissue slides were cut, deparaffinized in xylene, and rehydrated in a graded series of alcohol. Slides were treated in a PreTreatment module (Lab Vision Corp., UK Ltd, UK) in Tris-HCl buffer (pH 8.5). Blocking of endogenous peroxidase was carried out with 0.3% Dako REAL Peroxidase-Blocking Solution.

The antibodies used in immunostaining were: polyclonal rabbit anti-human TLR 2, 3, 4 (1:50, sc-10739, sc-10740, sc-10741), and 9 (1:100, sc-25468 Santa Cruz Biotechnology, USA), monoclonal mouse anti-human TLR 5 (1:200, IMG-664A Imgenex, USA), monoclonal rabbit anti-human TLR 7 (1:300, IMG-581A Imgenex, USA), and monoclonal mouse anti-human p16^{INK4a} (9517 CINTec Histology Kit, MTM laboratories, Germany).

Epitope retrieval was carried out with Tris-HCl for TLR 2, 4, and 7, and Tris-EDTA for TLR 3, 5, and 9. Dako REAL DAB+ Chromogen was used for staining

visualization. Between each step, PBS-0.04%-Tween20 was used for washing the slides. Counterstaining was performed with Meyer's hematoxylin and mounting with Aquamount, (BDH, Poole, UK). Antibody incubation was carried out with the Dako REAL Antibody Diluent S2022. The incubation time was 60 minutes for TLR antibodies and 30 minutes for p16^{INK4a} antibodies. For each antibody, a positive control was used. Incubation of negative controls was performed in diluent lacking the primary antibody.

8.1.6 Cell culturing and immunoblotting

UT-SCC cells were cultured at 37°C in DMEM medium with 4.5 g/l glucose, supplemented with 10% fetal calf serum (FCS), HEPES, non-essential amino acids, glutamine, and penicillin and streptomycin (Sigma Aldrich). The HSC-3 cells were cultured in an equivalent amount of DMEM medium with 4.5 g/l glucose and Ham's F12 Nutrient mixture supplemented with 10% FCS, penicillin, streptomycin, sodium pyruvate, and hydrocortisone. Nonidet P-40 (NP-40) lysis buffer (1% NP-40, 20 mM HEPES, pH 7.5, 150 mM NaCl) was used in the lysing of cells supplemented with 50 mM NaF, 1 mM Na₃VO₄ and 1 x Complete Proteinase Inhibitor Cocktail (Roche, Basel, Switzerland) at 4°C for 30 min. Centrifugation was used for the removal of detergent-insoluble material (16 000 x g at 4 °C for 15 min).

SDS-PAGE (10%) served in separation, PVDF-FL membranes (Millipore, Billerica, MA) served as transfer membrane, and Odyssey blocking buffer (LI-COR, Lincoln, NE) diluted 1:1 with PBS served in blocking of equivalent amounts of total cell lysates (50 µg). Membranes were incubated with TLR 5 and 7 antibodies as described above, mouse anti-actin (Sigma) followed by Alexa Fluor 680 (Invitrogen) and IRDye 800 (LI-COR Biosciences) anti-mouse or anti-rabbit secondary antibodies. The dilution of 1:200 was used for TLR 5 and 7 antibodies. The Odyssey Infrared Imager (LI-COR) was used in signal detection and Odyssey software in subsequent quantification.

8.1.7 HPV assays

In Study III, we detected HPV-DNA by PCR. DNA was extracted from from the paraffin embedded formalin-fixed sections and amplified with primer sets 1 and 2 from the Multiplex HPV Genotyping Kit® (DiaMEX GmbH, Germany). A negative control, which contained no genomic DNA, was used to confirm the absence of contamination. Genotyping of HPV was performed with a Multiplex HPV Genotyping Kit® (DiaMEX GmbH, Germany) detecting the following 24 LR- and HR-HPV-genotypes: LR-HPV6, 11, 42, 43, 44, and 70; and HR-HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82 (218). A Luminex LX-100 analyzer (Bio-Plex 200 System, Bio-Rad Laboratories, Hercules, USA) served in the analysis of the labeled hybrids.

Pellets of UT-SCC-65, -69, -60A, -60B, and -102 cells were formed, and they were analyzed at Quattromed HTI Laboratory, Tartu, Estonia. HPV DNA amplification was performed targeting the E6/E7 region of the viral genome by PCR. Products were detected using a laboratory-developed Luminex-based assay (Quattromed).

In Study IV, we used the Ventana Inform HR-HPV *in situ* hybridization assay with a high-risk HPV probe and iVIEW Blue detection kit and the Benchmark XT series stainer (Tuscon, Arizona, USA). Tissues sectioned at 5 µm thickness were used. Extended Ventana cell conditioning solution (CC2) was used and pretreatment was carried out with an incubation time of 32 minutes with ISH protease 3. In the assay, the following HR-HPV subtypes have been demonstrated to be detected: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66.

8.1.8 Statistical analysis

Statistical analysis was performed in SPSS Version 20.0 (SPSS, Inc., Chicago, IL, USA). The chi-square test with asymptotic and exact *P* values and the Mann-Whitney U-test were used in calculating the statistical significance of differences between categorical variables. The differences in continuous variables between independent groups were evaluated using the independent-samples T-test and the Mann-Whitney U-test. The normality of the distribution was inspected from a histogram, and in uncertain cases, the Shapiro-Wilk test served in the evaluating the normality of the distribution. The Kaplan-Meier estimate with the log-rank test and Cox proportional hazards model were used to analyze the survival of patients according to clinicopathological factors and biomarkers. The endpoints were as follows: overall survival (OS), disease-specific survival (DSS), disease-free survival (DFS), and recurrence-free survival (RFS). In order to minimize follow-up bias, we adjusted the follow-up time in the analysis to a maximum of five years. The follow-up time was the period between the last day of treatment and the end of follow-up or death of any cause in OS, and death with disease in DSS. The DFS time was calculated from the last treatment day to the detection of OPSCC recurrence either at the primary tumor site, in regional lymph nodes, or both, or at distant sites. In addition to recurrence, death of any cause was considered as an event. The RFS time was similarly calculated from the last treatment day to the detection of OPSCC recurrence either at the primary tumor site, in regional lymph nodes, or both or, at distant sites. In order to adequately present the recurrence rate of locoregional and distant RFS, we only considered OPSCC recurrence as an event. A two-tailed *P*-value of less than 0.05 was considered statistically significant.

8.1.9 Ethical considerations

This study obtained institutional study permission and approval from the local ethics committee (record number: 179/13/03/02/2013). In Study I, all university hospitals obtained their own institutional study permission.

9. RESULTS

9.1 TREATMENT AND OUTCOME OF OROPHARYNGEAL CARCINOMA IN FINLAND (STUDY I)

In total, 674 patients were diagnosed and treated for OPSCC at the five Finnish university hospitals over the ten-year period from 2000–2009. Most of the patients (n = 313, 46.4%) were from the Helsinki University Hospital area. Of these patients, 600 received treatment with curative intent, and their clinical characteristics are presented in Table 3. The mean age of the patients was 58.4 years (range 26.5–90.8 years). Patients with a p16-positive tumor were younger, with a mean age of 56.8 years, than those carrying a p16-negative tumor, with a mean age of 60.6 years. The lateral wall of the OP was the most common subsite of primary tumors, and p16 positivity was clearly associated with this subsite. In the anterior wall of the OP, the proportion of p16-positive and -negative tumors was almost equal. Superior-wall tumors were mostly p16 negative, and posterior-wall tumors rarely occurred. p16-positive tumors were somewhat more often classified as T1-2 (66.3%) than p16-negative tumors (54.2%), but the classification of regional lymph nodes in p16-positive disease was higher (cN+ 84.9%) than in p16-negative diseases (cN+ 68.7%). Therefore, the stage was generally higher in the p16-positive group (III-IV 90.1%) than in the p16-negative group (III-IV 76.5%). Smoking habits also differed between these groups, as among patients with a p16-negative tumor, only 3.5% were never-smokers, while the corresponding proportion was 37.4% in p16-positive group. Most tumors that were p16 negative were well or moderately differentiated (grade 1–2, 67.3%), while 61.6% of p16-positive tumors were poorly differentiated.

Of the 600 patients with curative treatment intent, 564 were able to receive the complete treatment. Of this patient group, 270 (47.9%) received primary surgery. ND was performed for 243 (92.4%) at the time of primary surgery. Postoperative oncological treatment was given to 248 (91.9%) patients. Oncological treatment as curative first-line treatment was given to 294 (52.1%) patients. Of these, 249 (84.7%) received CRT and 45 (15.3%) RT. Three patients received RT with adjuvant or neoadjuvant CT. Surgery after definitive CRT and RT was performed for 46 and 8 patients, respectively.

The 3-year OS, DSS, and DFS in all patients treated with curative intent was 70.2%, 76.7%, and 66.0%, respectively. The 3-year DSS in patients with a p16-positive tumor was 88.1% and with a p16-negative tumor 63.2%. The treatment received and the 3-year outcome for the patients treated with curative intent for lateral-wall and anterior-wall tumors are presented in Table 4.

Table 3. Baseline clinical characteristics of the 600 oropharyngeal squamous cell carcinoma patients treated with curative intent. p16 staining was available from 431 patients.

| | All patients n=600 | p16 positive n=252 | p16 negative n=179 | <i>P</i> |
|-----------------|-----------------------|-----------------------|-----------------------|-----------|
| Sex | | | | 0.745* |
| Men | 442 | 188 | 136 | |
| Women | 158 | 64 | 43 | |
| Age (yrs. mean) | 58.4 | 56.8 | 60.6 | <0.001** |
| Localization | | | | <0.001*** |
| Lateral | 387 | 186 | 86 | |
| Anterior | 152 | 60 | 58 | |
| Superior | 47 | 4 | 29 | |
| Posterior | 14 | 2 | 6 | |
| T class | | | | 0.011* |
| T1-T2 | 363 | 167 | 97 | |
| T3-T4 | 237 | 85 | 82 | |
| N class | | | | <0.001* |
| N0 | 142 | 38 | 56 | |
| N+ | 458 | 214 | 123 | |
| Stage | | | | <0.001* |
| I-II | 96 | 25 | 42 | |
| III-IV | 504 | 227 | 137 | |
| Smoking | | | | <0.001* |
| Never | 118 | 77 | 5 | |
| Earlier | 134 | 74 | 25 | |
| Current | 227 | 55 | 113 | |
| Unknown | 121 | N/A | N/A | |
| Grade | | | | <0.001* |
| 1 | 49 | 12 | 25 | |
| 2 | 188 | 66 | 74 | |
| 3 | 223 | 125 | 48 | |
| Unknown | 140 | N/A | N/A | |

*=Chi-square test with asymptotic *P*-value, **Independent samples t-test

***=Chi-square test with exact *P*-value

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According to the univariate analysis, patients who underwent primary surgery and received postoperative oncological treatment for lateral-wall OPSCC had better DSS than those who received definitive oncological treatment. Patients with anterior-wall OPSCC had better DSS if they received definitive CRT instead of other treatment.

In patients with p16-positive OPSCC, the 3-year DSS rate for those patients who underwent definitive CRT was 86.3%, compared with 93.4% in those patients who underwent upfront surgery followed by adjuvant treatment ($p = 0.077$). The corresponding figures for p16-negative OPSCC were 67.7% and 68.9% ($p = 0.621$). Among currently smoking patients, the 3-year DSS was lower in patients treated with definitive CRT (62.9%) compared with those who underwent upfront surgery followed by adjuvant treatment (75.8%), but the difference was not statistically significant ($p = 0.066$). More importantly, among smokers with a p16-positive primary tumor, the corresponding figures were (72.7%) and (91.6%) ($p = 0.028$). Among smokers with a p16-positive primary tumor, there was no association between the treatment method and DSS. (Jouhi, L et al., unpublished results)

Table 4. The 3-year outcome in lateral-wall (tonsillar and tonsillar pillars) and anterior-wall (base of tongue and vallecula) oropharyngeal squamous cell carcinoma according to treatment

| | No. of patients | OS(%) | DSS(%) | DFS(%) |
|------------------------------|-----------------|-------|--------|--------|
| Lateral-wall disease | | | | |
| All | 387 | 75.8 | 81.5 | 75.5 |
| Sx | 17 | 47.1 | 50.7 | 25.3 |
| Sx+RT | 105 | 78.9 | 86.3 | 77.0 |
| Sx+CRT | 91 | 87.7 | 93.0 | 85.5 |
| RT | 29 | 51.7 | 59.3 | 48.3 |
| RT + Salvage Sx | 8 | 62.5 | 62.5 | 62.5 |
| CRT | 88 | 73.7 | 79.0 | 65.6 |
| CRT + Salvage Sx | 46 | 80.4 | 84.4 | 71.7 |
| Anterior-wall disease | | | | |
| All | 152 | 59.2 | 66.9 | 28.6 |
| Sx | 14 | 35.7 | 42.9 | 35.7 |
| Sx+RT | 26 | 57.7 | 66.8 | 57.7 |
| Sx+CRT | 11 | 63.6 | 63.6 | 63.6 |
| RT | 5 | 0 | 20.0 | 0 |
| RT + Salvage Sx | 2 | 50.0 | 50.0 | 50.0 |
| CRT | 65 | 69.2 | 77.1 | 64.6 |
| CRT + Salvage Sx | 29 | 58.6 | 68.1 | 58.6 |

OS = Overall survival, DSS = Disease-specific survival, DFS = Disease-free survival, Sx = Surgery, RT = Radiotherapy, CRT = Chemoradiotherapy
Three patients treated with definitive RT and adjuvant or neoadjuvant chemotherapy are not presented.

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Multivariate analysis of patients treated with curative intent was performed separately for lateral-wall and anterior-wall OPSCC, because the tumor site had a statistically significant interaction with treatment ($p = 0.013$), with RT modality ($p = 0.001$), and with N class ($p = 0.037$) (Table 5). Factors associated with disease mortality in lateral-wall disease were the presence of cervical metastasis, p16 negativity, and male sex. Among patients with anterior-wall disease, p16 negativity, male sex, classes T3-T4, and 3D conformal RT were factors associated with a higher likelihood of disease mortality.

Table 5. Multivariate Cox regression analysis for the 5-year disease specific survival of patients with lateral-wall (tonsillar and tonsillar pillars) and anterior-wall (base of tongue and vallecula) oropharyngeal squamous cell carcinoma

| | | Disease-specific survival | | |
|--------------------------------|-----|---------------------------|-----|----------|
| | | <i>P</i> | HR | 95% CI |
| Lateral wall | | | | |
| No. of patients | 224 | | | |
| Age | | | | |
| Male vs Female | | 0.016 | 5.8 | 1.4–24.2 |
| T3-4 vs T1-2 | | | | |
| N+ vs N0 | | 0.029 | 9.2 | 1.2–68.0 |
| p16- vs p16+ | | <0.001 | 4.4 | 2.3–8.5 |
| CRT ± Salvage Sx vs Sx + (C)RT | | 0.073 | 1.8 | 0.9–3.5 |
| Anterior wall | | | | |
| No. of patients | 98 | | | |
| Male vs Female | | 0.028 | 3.9 | 1.2–13.5 |
| T3-4 vs T1-2 | | 0.016 | 2.7 | 1.2–12.5 |
| p16- vs p16+ | | 0.021 | 2.6 | 1.2–5.7 |
| 3D vs IMRT | | <0.001 | 5.5 | 2.4–2.5 |

All models are adjusted for age, sex, T class, N class, p16, treatment (Sx + (C)RT = Surgery + (chemo)radiotherapy vs. CRT ± Sx = Chemoradiotherapy ± Salvage surgery), and radiotherapy modality (3D = 3D conformal radiotherapy vs IMRT = Intensity-modulated radiotherapy), HR = Hazard ratio, CI = Confidence interval

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9.2 MANAGEMENT OF N+ NECK AND REGIONAL RECURRENCES (STUDY II)

In Study II, the incidence of RRs was evaluated in 169 patients with complete curative treatment for cN+ OPSCC and a curative response after three months from treatment completion. Of these, 107 patients underwent primarily surgical treatment and 62 received definitive oncological treatment. Altogether, seven RRs occurred: five in the primarily surgically treated group and two in the definitive oncological treatment group. The incidence of RR was low in both groups (4.7% and 3.2%), and despite the treatment change towards a more oncological approach during the study period, the incidence of RRs remained unchanged. Patients with RR are presented in Table 6. All patients who developed RR had clinical N class of N2b or higher. Six of the seven patients with RR had a p16-positive primary tumor. Regional recurrence developed in the contralateral side of the neck in five of the seven patients.

Table 6. Patients with regional recurrent disease

| Patient | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|--------------------------------------|----------------|-------------|---------------|---------------|---------------|---------------|----------------|
| Age | 45 | 58 | 78 | 72 | 77 | 66 | 64 |
| Sex | Male | Male | Male | Male | Male | Female | Male |
| Localization of primary tumor | Base of tongue | Tonsil | Tonsil | Vallecula | Tonsil | Tonsil | Base of tongue |
| Side of primary tumor | Bilateral | Unilateral | Unilateral | Bilateral | Unilateral | Unilateral | Unilateral |
| T class | T3 | T3 | T4a | T3 | T2 | T1 | T1 |
| N class | N2b | N2b | N2b | N2c | N2b | N2b (pN0) | N2b |
| p16 status of primary tumor | - | + | + | + | + | + | + |
| Primary treatment | CRT | Sx + RT | Sx + RT | CRT | Sx + RT | Sx | Sx + CRT |
| Neck dissection | No | Ipsilateral | Ipsilateral | No | Ipsilateral | Ipsilateral | Ipsilateral |
| Neck radiotherapy | Bilateral | Bilateral | Ipsilateral | Bilateral | Bilateral | No | Bilateral |
| Time to regional failure (mo) | 6 | 16 | 6 | 25 | 22 | 11 | 3 |
| Recurrence side | Contralateral | Ipsilateral | Contralateral | Contralateral | Contralateral | Contralateral | Ipsilateral |
| p16 status of recurrence | - | + | - | + | + | + | + |
| Treatment of recurrence | No | Salvage | Salvage | Salvage | Salvage | Salvage | Salvage |
| Outcome (mo) | Dead (5) | Dead (3) | Dead (23) | Alive (110) | Dead (41) | Alive (180) | Dead (47) |

CRT = Chemoradiotherapy, Sx = Surgery, RT = Radiotherapy

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9.3 EXPRESSION OF TOLL-LIKE RECEPTORS IN HR-HPV-POSITIVE AND HR-HPV-NEGATIVE OPSCC *IN VIVO* AND *IN VITRO* (STUDY III)

Tissue samples were collected from the most recent OPSCC cases (n = 35) for which p16 status was available. Twenty-one of the samples were positive for p16 IHC and 14 were negative. HR-HPV positivity was detected in 18 samples, while 17 were HR-HPV negative. The sensitivity and specificity of p16 for HR-HPV detection was 94.4% and 76.5%, respectively. All the investigated TLRs

(TLR 2, 3, 4, 5, 7, and 9) were found to be expressed in OPSCC, and TLRs 2, 3, 4, 7, and 9 were expressed in tumor-infiltrating lymphocytes. TLR 7 and TLR 9 expression patterns depended on the tumor HPV status: TLR 7 expression was high and TLR 9 expression low in HR-HPV-positive OPSCC. The expression patterns of TLR 5 and 7 depended on the tumor p16 status: TLR 5 expression was low and TLR 7 expression high in p16-positive OPSCC. (Table 7).

Table 7. The expression of toll-like receptor 2, 3, 4, 5, 7, and 9 in HPV-positive and HPV-negative OPSCC and in p16-positive and p16-negative OPSCC

| | HPV-positive OPSCC n=18 | | | | HPV-negative OPSCC n=17 | | | | P | Missing |
|---------------------------------|-------------------------|---|----|----|-------------------------|----|----|---|--------|---------|
| | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 | | |
| Immunopositivity | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 | | |
| TLR 2 Cytoplasm | 0 | 3 | 9 | 6 | 1 | 5 | 6 | 5 | 0.405 | 0 |
| TLR 3 Membrane & Cytoplasm | 0 | 3 | 5 | 10 | 0 | 1 | 12 | 4 | 0.273 | 0 |
| TLR 4 Cytoplasm | 2 | 4 | 8 | 3 | 3 | 9 | 2 | 3 | 0.369 | 1 |
| TLR 5 Membrane | 12 | 2 | 3 | 1 | 5 | 5 | 4 | 3 | 0.062 | 0 |
| TLR 5 Cytoplasm | 13 | 2 | 2 | 1 | 6 | 5 | 3 | 3 | 0.062 | 0 |
| TLR 7 Nuclear membrane & Nuclei | - | 1 | 11 | 6 | - | 13 | 3 | 1 | <0.001 | 0 |
| TLR 9 Membrane & Cytoplasm | 3 | 9 | 3 | 2 | 0 | 4 | 10 | 3 | 0.018 | 1 |
| | p16-positive OPSCC n=21 | | | | p16-negative OPSCC n=14 | | | | | |
| TLR 2 Cytoplasm | 0 | 5 | 10 | 6 | 1 | 3 | 5 | 5 | 1.000 | 0 |
| TLR 3 Membrane & Cytoplasm | 0 | 3 | 8 | 10 | 0 | 1 | 9 | 4 | 0.538 | 0 |
| TLR 4 Cytoplasm | 4 | 6 | 7 | 3 | 1 | 7 | 3 | 3 | 0.823 | 1 |
| TLR 5 Membrane | 14 | 3 | 3 | 1 | 3 | 4 | 4 | 3 | 0.015 | 0 |
| TLR 5 Cytoplasm | 16 | 2 | 2 | 1 | 3 | 5 | 3 | 3 | 0.006 | 0 |
| TLR 7 Nuclear membrane & Nuclei | - | 3 | 12 | 6 | - | 11 | 2 | 1 | 0.001 | 0 |
| TLR 9 Membrane & Cytoplasm | 3 | 8 | 6 | 3 | 0 | 5 | 7 | 2 | 0.306 | 1 |

The Mann-Whitney U test was used in statistical comparisons

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Five OPSCC cell lines (UT-SCC 60A, 60B, 65, 69, and 102) were examined for p16 status, HR-HPV status, and TLR 5 and 7 expressions. The oral SCC cell line HSC-3, known to be HR-HPV negative, was studied for p16 status and TLR 5 and 7 expressions. All five OPSCC cell lines were HR-HPV-positive, but only one, UT-SCC 69, was p16 positive, whereas the rest (UT-SCC 60A, 60B, 65, 69, 102, and HSC-3) were p16 negative. The cell line UT-SCC 69 expressed less TLR 5 and more TLR 7 compared with the p16-negative OPSCC cell lines. In addition, UT-SCC 69 expressed less TLR 5 than HSC-3, although the expression levels of TLR 7 did not differ significantly.

9.4 TOLL-LIKE RECEPTORS 5, 7, AND 9 AND PROGNOSIS IN OPSCC (STUDY IV)

Altogether, 202 patients receiving treatment with curative intent for first-primary OPSCC had primary tumor samples available for TLR 5, TLR 7, TLR 9, and p16 expression and HPV status determination. Baseline clinicopathological data, as well as TLR 5 and 7 expressions in relation to HR-HPV status and p16 status are provided in Table 8. Compared with HR-HPV-negative tumors, HR-HPV-positive tumors expressed lower levels of TLR 5 and TLR 9 and higher levels of TLR 7.

High TLR 5 expression and low TLR 7 expression were associated with poor DSS (Figure 3). In the subgroup of HPV-positive tumors, patients with high TLR 5 expression and low TLR 7 expression had poor DSS, but among patients with HPV-negative OPSCC, the expression of these TLRs had no impact on DSS. In the Cox proportional hazards model, we adjusted the TLR 5 and 7 expressions for sex, T class, N class, and HR-HPV (Table 9). HPV status had a statistically significant interaction with TLR 7 expression ($p = 0.027$), leading to stratification of multivariate analysis according to HR-HPV status. Among patients with HPV-positive tumors, high TLR 5 expression and low TLR 7 expression presented as independent factors for poor DSS. In the HR-HPV-negative subgroup, males and patients with advanced regional metastasis (N2a-N3) had significantly increased risk of disease mortality.

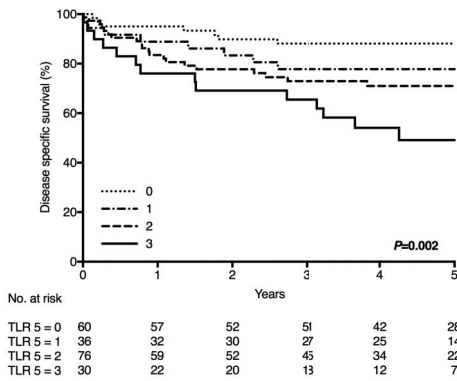
Table 8. Baseline clinicopathological data of patients and the expression of TLR 5, 7, and 9 in relation to HPV and p16 status

| | HPV+ | HPV- | P | p16+ | p16- | P | Missing |
|------------------------|-----------|-----------|----------|------------|-----------|----------|---------|
| Sex | | | | | | | |
| Men | 81 (54.0) | 69 (46.0) | | 89 (59.3) | 61 (40.7) | | |
| Women | 24 (46.2) | 28 (53.8) | 0.329* | 28 (53.8) | 24 (46.2) | 0.490* | 0 |
| Age^a | | | | | | | |
| Lowest 1/3 | 37 (55.2) | 30 (44.8) | | 41 (61.2) | 26 (38.8) | | |
| Middle 1/3 | 37 (54.4) | 31 (45.6) | | 43 (63.2) | 25 (36.8) | | |
| Highest 1/3 | 31 (46.3) | 36 (53.7) | 0.301* | 33 (49.3) | 34 (50.7) | 0.163* | 0 |
| Smoking | | | | | | | |
| Never | 24 (92.3) | 2 (7.7) | | 24 (92.3) | 2 (7.7) | | |
| Earlier | 35 (71.4) | 14 (28.6) | | 39 (79.6) | 10 (20.4) | | |
| Currently | 26 (27.1) | 70 (72.9) | <0.001* | 31 (32.3) | 65 (67.7) | <0.001* | 32 |
| Alcohol abuse | | | | | | | |
| Never | 41 (67.2) | 20 (32.8) | | 41 (67.2) | 20 (32.8) | | |
| Earlier | 6 (25.0) | 18 (75.0) | | 9 (37.5) | 15 (62.5) | | |
| Currently | 9 (23.7) | 29 (76.3) | <0.001* | 12 (31.6) | 26 (68.4) | <0.001* | 72 |
| T class | | | | | | | |
| T1 | 20 (52.6) | 18 (47.4) | | 24 (63.2) | 14 (36.8) | | |
| T2 | 38 (50.0) | 38 (50.0) | | 45 (59.2) | 31 (40.8) | | |
| T3 | 25 (54.3) | 21 (45.7) | | 24 (57.2) | 22 (47.8) | | |
| T4 | 22 (52.4) | 20 (47.6) | 0.871* | 24 (57.1) | 18 (42.9) | 0.461* | 0 |
| N class | | | | | | | |
| N0-N1 | 27 (40.9) | 39 (59.1) | | 32 (48.5) | 34 (51.5) | | |
| N2-N3 | 78 (57.4) | 53 (42.6) | 0.029* | 85 (62.5) | 51 (37.5) | 0.059* | 0 |
| Stage | | | | | | | |
| I-II | 10 (33.3) | 20 (66.7) | | 14 (46.7) | 16 (53.3) | | |
| III-IV | 95 (55.2) | 77 (44.8) | 0.027* | 103 (59.9) | 69 (40.1) | 0.177* | 0 |
| Tumor site | | | | | | | |
| Lateral wall | 77 (65.8) | 40 (34.2) | | 81 (69.2) | 36 (30.8) | | |
| Anterior wall | 25 (41.0) | 36 (59.0) | | 31 (50.8) | 30 (49.2) | | |
| Posterior wall | 0 (0.0) | 3 (100.0) | | 1 (33.3) | 2 (66.7) | | |
| Superior wall | 3 (14.3) | 18 (85.7) | <0.001** | 4 (19.0) | 17 (81.0) | <0.001** | 0 |
| Grade | | | | | | | |
| 1 | 3 (16.7) | 15 (83.3) | | 4 (22.2) | 14 (77.8) | | |
| 2 | 32 (41.0) | 46 (59.0) | | 39 (50.0) | 39 (50.0) | | |
| 3 | 70 (66.0) | 36 (34.0) | <0.001* | 74 (69.8) | 32 (30.2) | <0.001* | 0 |
| TLR 5 | | | | | | | |
| 0 | 47 (79.7) | 12 (20.3) | | 49 (83.1) | 10 (16.9) | | |
| 1 | 27 (73.0) | 10 (27.0) | | 32 (86.5) | 5 (13.5) | | |
| 2 | 23 (30.3) | 53 (69.7) | | 26 (34.2) | 50 (65.8) | | |
| 3 | 8 (26.7) | 22 (73.3) | <0.001* | 10 (33.3) | 20 (66.7) | <0.001* | 0 |
| TLR 7 | | | | | | | |
| 1 | 6 (12.8) | 41 (87.2) | | 6 (12.8) | 41 (87.2) | | |
| 2 | 44 (51.2) | 42 (48.8) | | 49 (57.0) | 37 (43.0) | | |
| 3 | 55 (79.7) | 14 (20.3) | <0.001* | 62 (89.9) | 7 (10.1) | <0.001* | 0 |
| TLR 9 | | | | | | | |
| 0 | 7 (77.8) | 2 (22.2) | | 6 (66.7) | 3 (33.3) | | |
| 1 | 23 (76.7) | 7 (23.3) | | 26 (86.7) | 4 (13.3) | | |
| 2 | 53 (57.6) | 39 (42.4) | | 57 (62.0) | 35 (38.0) | | |
| 3 | 22 (31.0) | 49 (69.0) | <0.001* | 28 (39.4) | 43 (60.6) | <0.001** | 0 |

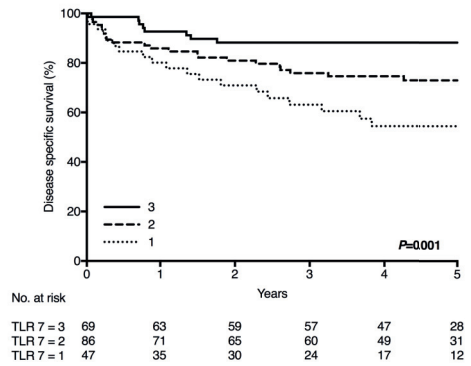
^aAge groups: lowest 1/3 ≤54.4 yrs. Middle 1/3 >54.4, <61.6 yrs. Highest 1/3 ≥61.1 yrs., *Chi-square test, asymptotic P-value, **Chi-square test, exact P-value, Lateral wall = tonsils and tonsillar pillars, Anterior wall = base of tongue and vallecula, Superior wall = soft palate and uvula

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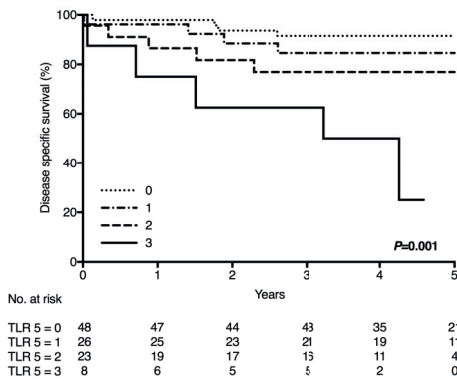
A. All patients according to TLR 5 expression



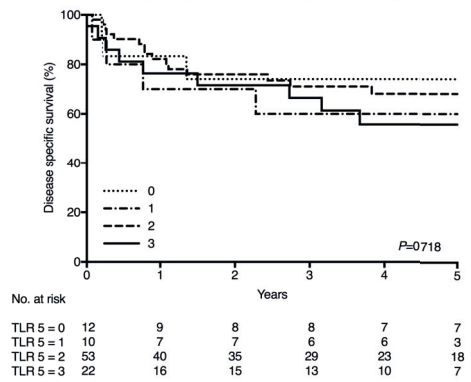
B. All patients according to TLR 7 expression



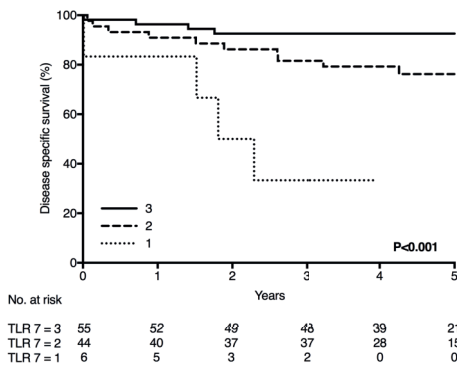
C. HPV-positive patients according to TLR 5 expression



D. HPV-negative patients according to TLR 5 expression



E. HPV-positive patients according to TLR 7 expression



F. HPV-negative patients according to TLR 7 expression

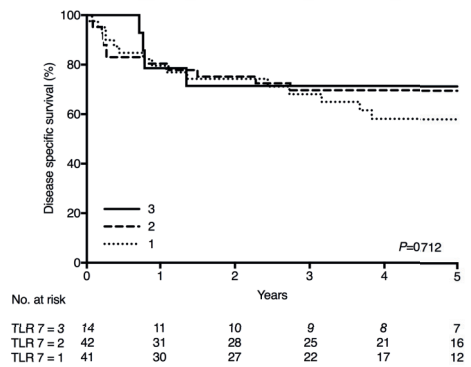


Figure 3. Disease-specific survival (DSS) curves according to TLR 5 and 7 expression in OPSCC, and also separately for HR-HPV-positive and HR-HPV-negative OPSCC. A: TLR 5 expression in OPSCC. High expression is associated with poor DSS. B: TLR 7 expression in OPSCC. High expression is associated with good DSS. C: TLR 5 expression in HR-HPV-positive OPSCC. High expression is associated with poor DSS, whereas low expression is associated with good DSS. D: TLR 5 expression in HR-HPV-negative OPSCC. No impact on DSS. E: TLR 7 expression in HR-HPV-positive OPSCC. High expression is associated with good DSS, whereas low expression is associated with poor DSS. F: TLR 7 expression in HR-HPV-negative OPSCC. No impact on DSS.

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Table 9. Multivariate Cox regression analysis for DSS of all OPSCC patients and separately for patients with HPV-positive tumors and HPV-negative tumors

| | Multivariate analysis | | | Multivariate analysis | | | Multivariate analysis | | | |
|-----------------|-----------------------|----------------|--------------|-----------------------|------------------|------------------|-----------------------|----------------|--------------|--|
| | All patients | | | HPV-positive patients | | | HPV-negative patients | | | |
| | HR | 95% CI | P | HR | 95% CI | P | HR | 95% CI | P | |
| Sex | | | | | | | | | | |
| Male vs. Female | 2.2 | 1.0-5.0 | 0.061 | 1.1 | 0.3-4.2 | 0.874 | 3.4 | 1.1-10.4 | 0.029 | |
| T class | | | 0.063 | | | 0.235 | | | 0.266 | |
| T2 vs. T1 | 2.0 | 0.7-5.8 | 0.174 | 2.3 | 0.2-21.9 | 0.463 | 2.3 | 0.7-7.6 | 0.188 | |
| T3 vs. T1 | 1.2 | 0.4-3.6 | 0.747 | 2.2 | 0.2-22.9 | 0.511 | 1.0 | 0.3-3.8 | 0.980 | |
| T4 vs. T1 | 3.1 | 1.1-8.9 | 0.031 | 5.5 | 0.6-48.5 | 0.122 | 2.4 | 0.7-8.4 | 0.169 | |
| N class | | | | | | | | | | |
| N2-3 vs. N0-1 | 3.6 | 1.7-7.5 | 0.001 | 4.0 | 0.8-19.2 | 0.084 | 3.4 | 1.4-8.2 | 0.007 | |
| TLR 5 | | | 0.228 | | | 0.033 | | | 0.761 | |
| 1 vs. 0 | 2.0 | 0.7-5.7 | 0.169 | 3.6 | 0.7-19.3 | 0.129 | 2.1 | 0.4-9.5 | 0.353 | |
| 2 vs. 0 | 1.7 | 0.7-4.2 | 0.280 | 2.4 | 0.6-10.2 | 0.240 | 1.4 | 0.4-5.2 | 0.594 | |
| 3 vs. 0 | 2.8 | 1.0-7.8 | 0.051 | 11.7 | 2.1-63.6 | 0.005 | 1.8 | 0.4-7.8 | 0.405 | |
| TLR 7 | | | 0.085 | | | 0.001 | | | 0.739 | |
| 1 vs. 3 | 3.2 | 1.1-9.0 | 0.027 | 20.8 | 4.0-107.1 | <0.001 | 1.6 | 0.4-6.0 | 0.471 | |
| 2 vs. 3 | 2.1 | 0.9-5.2 | 0.089 | 2.4 | 0.7-8.2 | 0.158 | 1.3 | 0.4-4.5 | 0.693 | |
| HPV | | | | | | | | | | |
| HPV- vs. HPV+ | 1.6 | 0.8-3.3 | 0.181 | | | | | | | |

HR = Hazard ratio, CI 95% = Confidence interval 95%

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10. DISCUSSION

The OP is currently the most important subsite for HNSCC research, because the incidence of OPSCC has rapidly increased. The disease has now been categorized into two distinct entities according to the p16 status. In addition, the treatment paradigm has changed towards a more oncological approach. Patients with HR-HPV-positive tumors have markedly better survival, but due to the increased incidence, half of all OPSCC recurrences develop in patients with a HR-HPV-positive tumor (17). Furthermore, the pattern of recurrences is different: The rate of locoregional recurrence in HR-HPV-negative OPSCC is higher than in HR-HPV-positive OPSCC, but the rate of distant recurrence does not differ significantly between the two (14, 172). Reliable information regarding the optimal treatment for OPSCC remains limited, and differences between national OPSCC treatment guidelines possibly reflect this fact. Advances in the survival stratification for HR-HPV-positive and HR-HPV-negative OPSCC, which may be offered by the new UICC and AJCC 8th edition (24, 25), might benefit the treatment selection. It is to be expected that the treatment for these two entities will become different. This study evaluated the OPSCC outcome in Finland, treatment of the cN+ neck, as well as the expression and prognostic value of TLRs in OPSCC.

9.5 OROPHARYNGEAL CARCINOMA IN FINLAND: CHANGING TREATMENT AND IMPROVED OUTCOME (STUDY I)

This study described the treatment provided and factors influencing the outcome in a nationwide framework over a 10-year period. The incidence of OPSCC in Finland has increased, and this trend is likely to be related to the increase in p16-positive OPSCC cases.

Among patients treated at Helsinki University Hospital, 18% had undergone tonsillectomy as a diagnostic procedure. Sometimes, tonsillectomy had resulted in clear margins. The intention in these cases was primarily a diagnostic procedure, and we therefore classified tonsillectomy as a biopsy in our study. Interestingly, in 19% of our patients, the tumor was classified as CUP before the diagnosis of OPSCC. Of these patients, 78% had a p16-positive primary tumor. (Jouhi, L et al., unpublished results)

This study also demonstrated that treatment has changed in Finland as compared with a previous Finnish nationwide retrospective study including all patients diagnosed and treated for OPSCC at the same institutions during the years 1995–1999 (121). In the late 1990s, upfront surgery was the mainstay for treatment, as 85% of all patients underwent primary surgery (121), whereas

the corresponding figure was 49% among patients treated with curative intent in the present series. A similar paradigmatic change to that occurring in Finland has also been reported from other countries (28, 122). Definitive oncological treatment has been suggested as the first-line treatment in Denmark (30) and Sweden (29), although in the United Kingdom (20) and in the US (22), for instance, the national protocol does not similarly emphasize the role of non-surgical treatment. In our series, the treatment protocol seemed to function well, as 94% of the patients treated with curative intent were able to complete their treatment.

The treatment outcome has also undergone changes in Finland. Although the five-year DSS remained fairly similar between 1995–1999 and 2000–2009 (73% and 75%) in lateral-wall disease, the corresponding figures in anterior-wall disease were 47% and 65%. The positivity of p16 in anterior-wall tumors increased from 38% in 2000–2004 to 59% in 2005–2009. The factors associated with good DSS among patients with anterior-wall OPSCC were p16 positivity, female sex, lower T class (T1-T2), and IMRT. In lateral-wall OPSCC, the treatment outcome remained similar. One explanation for this phenomenon could be the fact that the rate of p16-positive tumors in that site increased only slightly during the study period, from 64% to 71%. The disease mortality in lateral-wall OPSCC was influenced by the presence of regional metastasis, p16, and sex. Patients treated with definitive CRT ± salvage surgery compared with those who underwent primary surgery + (C)RT had a HR of 1.8, but the finding remained non-significant ($p = 0.073$). The observation was significant in the model without backward elimination, however.

According to the results of this study the role of surgery in the management of tonsillar disease remains open. A prospective randomized controlled study is warranted to evaluate the treatment of tonsillar disease. It has been suggested that even non-radical surgery may improve survival among tonsillar SCC patients (219, 220). In base of tongue SCC, the treatment modality did not have any effect on survival in multivariate analysis. However, surgical methods such as TORS may benefit patients from a functional viewpoint: if primary tumors are resected, patients may require less intensive postoperative oncological therapy (76, 120). In our material, 87 patients had a T1-T2 classified anterior-wall tumor, of whom 36% underwent upfront surgery, typically requiring tissue transfers. These surgeries probably caused significant morbidity. TORS could especially benefit this patient group, but requires further study. Interestingly, according to our results, patients who underwent primary surgery did not benefit from clear surgical margins. Free margins are generally considered important in HNSCC treatment, as the margin status may impact on the outcome (221). Margins are, however, also important in HR-HPV-positive OPSCC: It has been suggested that in HPV-positive, but not in HR-HPV-negative OPSCC, positive margins would independently increase overall mortality (222).

Our data do not support the findings of Wang et al. (18) and Seikaly et al. (19), who reported that patients with p16-negative OPSCC had significantly worse survival if they were treated with definitive oncological treatment instead of upfront surgery followed by adjuvant therapy. Furthermore, Seikaly et al. (19) concluded that among smokers carrying either a p16-positive or a p16-negative tumor, higher survival rates would be achieved after upfront surgery than after definitive CRT. In our material, neither of these main treatment modalities appeared to be superior among patients with p16-negative OPSCC. However, among smokers with p16-positive OPSCC, upfront surgery followed by adjuvant treatment resulted in higher DSS rates than definitive CRT.

9.6 MANAGEMENT OF THE N+ NECK (STUDY II)

In the second study our objective was to analyze the treatment of the neck in OPSCC. We focused on cN+ disease only, whereas the treatment of cNo disease was not analyzed in our study. As the treatment paradigm has changed towards a more oncological approach, and surgery is principally adopted for salvage purposes the relative rate of ND has also decreased. During our study period at the Helsinki University Hospital, organ-preserving treatment was provided to patients at an increasing rate, although upfront surgery did not seem to decrease. We did not detect any trend towards an increased occurrence of RR, although organ preservation was pursued more often. Of the patients who underwent primary surgery, 4.7% developed a RR, and of those who underwent definitive oncological treatment, 3.2% developed RR. Therefore, a “scan and wait” policy may benefit patients and reduce treatment-related morbidity. The low rate of RRs after definitive oncological treatment (1.2% - 5%) has previously been reported (223-225). However, the rate of RRs has also been shown to be low (2%) after upfront surgery (226). Sakashita et al. investigated the benefit of upfront ND in cN+ OPSCC (227). Their study showed no regional control difference between the patient group that was treated with the primarily oncological treatment and the group that was treated with upfront ND.

RR constitutes a significant cause of death in OPSCC. According to a study by Viani et al., only 19% of patients were alive at 5 years after the detection of RR, while the corresponding figure was 31% after local recurrence (228). Similarly, Rösli et al. (229) reported lower survival rates after surgical salvage for regional recurrence than for local recurrence. It is noteworthy that in their series, most patients with a local recurrence did not undergo salvage surgery, possibly reflecting limited resectability. Likewise, in our material, five-year DSS among patients with isolated RR was 25.7%. To compare, in oral SCC, according to a study by Ebrahimi et al., in oral SCC, only 53% of patients who developed RR died of disease (230). Likewise, Ord et al. showed that the 3-

year OS after salvage surgery for RR in oral SCC was 50%. The outcome after salvage surgery in oral SCC appears to be impaired if ND has been performed prior to RR (231, 232). The pattern of recurrence in HR-HPV-positive OPSCC is different compared with HR-HPV-negative OPSCC: the rate of distant recurrences does not differ significantly between HR-HPV-positive and HR-HPV-negative OPSCC, but in HR-HPV-positive OPSCC, unlike in HR-HPV-negative OPSCC (172), locoregional recurrences are sparse, a phenomenon seen also in our nationwide cohort.

In our material, five out of the seven RRs occurred on the contralateral side of the neck. Notably, two of these occurred in patients with an ipsilateral tumor of the tonsil. Seikaly et al. (19) observed that contralateral neck disease is relatively common in OPSCC, and it is not related to the site of the primary tumor, but rather to T class and N class, as among patients with a T4 tumor or N2a+ disease, the risk of contralateral disease was increased. In their study, contralateral RRs occurred at a rate of 2% at five years (82). The corresponding figure in our study was 3%. In our study, only one patient who developed a RR had a T4 classified primary tumor, but all RRs occurred in patients having an N class of cN2b or higher.

9.7 THE EXPRESSION PATTERNS OF TOLL-LIKE RECEPTORS DIFFER BETWEEN HR-HPV-POSITIVE AND HR-HPV-NEGATIVE OROPHARYNGEAL CARCINOMA (STUDY III)

The third study evaluated the expression patterns of TLR 2, 3, 4, 5, 7, and 9 in OPSCC groups stratified by HR-HPV status and p16 expression. The expression of TLRs has been observed in a number of other HNSCCs (202, 206, 212, 233-236), but in the OP their expression and relationship with HR-HPV has remained unknown. TLRs have also been reported to have altered expression in carcinogenesis and carcinoma of cervix uteri (34, 35, 237). It has been shown that the expression patterns of TLRs differ between clearing and persisting HR-HPV infection of the cervical mucosa, possibly reflecting differences in the immune response between these events (33). In our material, TLR 5 expression was lower and TLR 7 expression higher in p16-positive OPSCC. Furthermore, TLR 7 expression was higher and TLR 9 expression lower in HPV-positive OPSCC. Kauppila et al. (202) studied TLR 5 expression in the oral epithelium and in oral tongue squamous cell carcinoma (OTSCC), and observed that TLR 5 was more often overexpressed in cancerous tissue compared to normal oral epithelium. In our study, TLR 5 was significantly more commonly expressed in HR-HPV-negative and p16-negative OPSCC compared with cancer-free tonsil epithelium. However, no significant difference in TLR 5 expression patterns existed between HR-HPV-positive or p16-positive OPSCC and cancer-free tissue. As it is well known that the importance of HR-HPV is minor in oral carcinoma (238), our results

suggest that OTSCC and HR-HPV-negative or p16-negative OPSCC share a similar TLR 5 expression profile. On the contrary, HR-HPV-positive and p16-positive OPSCC had a TLR 5 expression pattern closer to normal epithelium than to HR-HPV-negative OPSCC or OTSCC. This finding possibly reflects the comprehensive differences between HR-HPV-positive and HR-HPV-negative OPSCC (59, 60). However, TLR 7 overexpression has been reported in oral SCC (206), suggesting that in terms of TLR 7 expression HPV-negative OPSCC and oral SCC differ. A study by Hasan et al. demonstrated that TLR 9 expression is downregulated in HR-HPV-positive cervical carcinoma (35). In line with this finding, our results showed that HR-HPV-positive OPSCC had lower TLR 9 expression than its virus-negative counterparts. In the latest WHO atlas of head and neck tumors, p16-positive and p16-negative tumors are classified as different entities (69), which is in line with our TLR results.

9.8 TOLL-LIKE RECEPTORS 5 AND 7 MAY SERVE AS NOVEL PROGNOSTIC MARKERS IN HPV-POSITIVE OROPHARYNGEAL CARCINOMA (STUDY IV)

The fourth study determined the expression of TLR 5, 7, and 9 in a patient series covering all patients diagnosed and treated at Helsinki University Hospital over a ten-year period. The results concerning TLR 5, 7, and 9 expression in OPSCC stratified by HR-HPV and p16 status were similar to the previous study (Study III): the expression of these TLRs differed significantly between these two groups. In addition, TLR 5, 7, and 9 expressions were significantly associated with several patient or disease-related factors, most of which are also associated with HR-HPV status (6, 12, 14, 15, 239). Notably, high TLR 5 expression and low TLR 7 expression were associated with poor DSS. The patient cohort was analyzed separately for patients with a HR-HPV-positive and a HR-HPV-negative tumor, and these TLRs were only prognostic in the HR-HPV-positive subgroup.

An association between high TLR 5 expression and a poor prognosis in OTSCC has been reported (202), although contrary findings have also been presented (212). In addition, OSCC patients with high TLR 7 tumor expression have been shown to have a poor prognosis (206). In HR-HPV-negative OPSCC we did not observe any prognostic role of TLR 5 and 7, as suggested in OTSCC and OSCC.

Several attempts have been made to clarify the role of TLR 5 and 7 in various types of cancer. In gastric cancer and salivary gland adenocarcinoma, tumor progression has been shown to be associated with TLR 5 activation (240, 241). However, TLR 5 activation did not cause tumor progression in OSCC (242). TLR 5 activation with a synthetic agonist, CBLB502, mediated radioprotectivity in tissues adjacent to the tumor, but not in the tumor itself

(243). The authors speculated that the radioprotectivity occurred due to Nf- κ B-mediated antiapoptotic activity. According to their speculation, tumor tissues were not desensitized because of the constitutive Nf- κ B activity in cancer (244) or inhibition of TLR 5 downstream signaling due to the potential activation of PIK3 (245). PIK3 may in fact act as a negative regulator of TLR-mediated signals influencing inflammatory responses, cell proliferation and survival mediated by the Nf- κ B pathway (246). PIK3 activation is reported to promote radioresistance (247) and correspondingly inhibition of its downstream protein, AKT, is reported to promote radiosensitivity (248, 249). On the other hand, negativity of PTEN, a regulator of PIK3, combined with *PIK3CA* mutation may increase radiosensitivity in breast cancer (250). *PIK3CA*-activating mutations are relatively common in HR-HPV-positive OPSCC (66-68). (251) *PIK3CA* mutation has not reported to be prognostic in OPSCC (68) or in other HNSCCs (251). Amplification of PIK3 has been shown to be related to poor DFS in HNSCC patients with no lymph node metastasis (252). However, Sewell et al. reported that in their HR-HPV-positive cohort all disease deaths occurred among those patients, who did not have a *PIK3CA* mutation (66). In esophageal SCC *PIK3CA*-activating mutation has been shown to be related to a good prognosis (253). Therefore, the role of PIK3 appears to be contradictory in cancer. However, the association of the radiation response and TLR 5 expression remains speculative in HR-HPV-positive OPSCC. The association of TLR 5 and *PIK3CA* mutation, and their functions, especially regarding the radiation response, should be further examined in OPSCC.

Activation of TLR 7 has promoted treatment resistance in adenocarcinoma and SCC of the lung, possibly via an Nf- κ B mediated pathway and upregulation of antiapoptotic protein Bcl-2 (254, 255). Activation of TLR 7 in OSCC, however, induced tumor apoptosis and necrosis (256), and in breast cancer sensitized cancer cells to RT (257). Based on our results we may only hypothesize, whether the radiosensitivity of HR-HPV-positive tumors could be associated with TLR 7 overexpression.

Numerous potential prognostic or predictive molecular markers have been studied in HNSCC (258). Even though HR-HPV is well validated as a prognostic marker in OPSCC, the predictive value of HR-HPV requires further investigation (20-22). Therefore, further molecular marker studies in HNSCC are needed to find the biomarkers that could be used in daily practice.

9.9 STUDY STRENGTHS AND LIMITATIONS

Study I comprised a large cohort of consecutive patients treated over a 10-year period at the five Finnish University Hospitals, in which HNSCC treatment is mainly centralized. Unfortunately, we were unavailable to determine the

coverage of our patient series in comparison with the numbers provided by the Finnish Cancer Registry (FCR), because in its reports the pharynx has not been divided into its sublocations. According to the FCR, 1286 new pharyngeal cancer cases occurred during the time frame of this study. This number also includes pharyngeal carcinomas originating outside the OP, complicating head-to-head comparison. Thus, it remains speculative how large proportion of Finnish OPSCC patients were treated at the University Hospitals. However, our report offers comprehensive information on the treatment of OPSCC and the outcome after curative intent. More importantly, nearly all treatment for malignancies is performed at public hospitals in Finland, and treatment selection is not affected by factors such as income or insurance. In addition, the compliance with treatment is high and patient follow-up is comprehensive. The retrospective nature of our study caused limitations in data, especially concerning details on smoking and alcohol abuse. A fact that improves the feasibility of our results concerning the treatment outcome is that the p16 status had no effect on the treatment selection, but in contrast, a long inclusion period may have caused bias because treatment modalities have changed during the years. Our study would also have improved if we had been able to adjust for comorbidities and performance in the multivariate survival analysis. Therefore, we were not able to eliminate the impact of these factors in the treatment decision. In addition, our multivariate analysis suffered from moderate correlations between the investigated parameters, possibly reducing their explanatory power.

Study II included patients who were treated at Helsinki University Hospital and had N+ disease. As in Study I, treatment changed during the time frame, and the p16 status did not impact on treatment selection. However, we were unable to compare either the rate of complete treatment response or the occurrence of RR between the two treatment groups, as other factors, such as T and N classes and comorbidities, may have influenced treatment selection, and therefore biased the results. Nevertheless, we were able to show that the occurrence of RR was low, and while the treatment changed towards a more oncological approach, the incidence of RR did not increase.

Study III included a relatively small cohort of tumor samples, and it was not uniform regarding clinicopathological factors. No association was detected between TLR 5 expression and HPV status, or between TLR 9 expression and p16 status, possibly due to the small sample size. These associations were, however, observed in a larger cohort in Study IV. Although p16 status and HPV status were associated with each other *in vivo*, the results of the cell culture study revealed discordance between these factors. We may only speculate, whether the HR-HPV positivity in our p16-negative OPSCC cell lines reflects the actual involvement of HR-HPV in the disease etiology of these cell lines.

In Study IV we were able to demonstrate that TLR 5 and TLR 7 expression associated with DSS in HR-HPV-positive OPSCC. An important weakness in this study concerns the number of events. Although the cohort had 202 patients and the number of HR-HPV-positive cases was 105 (52.0%), the number of events in DSS analysis of this subgroup of interest was only 18 (17.1%), whereas it was 31 (32%) in the HR-HPV-negative group. A good prognosis in HPV-positive OPSCC is well-known from the literature, but it restricted the reliability of survival analyses, especially because the number of patients having adverse TLR expression was low. As a result of this, the 95% confidence intervals were broad for adverse TLR 5 and TLR 7 expression HRs. More importantly, the multivariate analysis stratified according to p16 status did not show a statistically significant HR for adverse TLR 7 expression, possibly due to the same reason. Therefore, robust conclusions cannot be drawn concerning TLR 5 and TLR 7 expression and survival, and further studies are needed for possible validation of these results. In addition, multivariate analysis suffered from a lack of knowledge of comorbidities and performance. The analyses included two patients whose tissue samples were obtained from salvage surgical specimens, in which the TLR expression before RT cannot be evaluated.

9.10 FUTURE ASPECTS

The treatment paradigm of OPSCC is undergoing continuous change, and the rate of surgery may again be increasing. Therefore, treatment and outcome details of OPSCC patients treated after 2009 would call for further evaluation in the future. A prospective database incorporated in patient records would markedly ease the evaluation of patient cohorts. Currently, p16 staining is routinely performed in Finland, but HR-HPV detection is not. A uniform method for HR-HPV status determination in clinical practice remains to be determined. Future research is also needed to find out whether the incidence of p16-positive OPSCC is still increasing. TORS was introduced for head and neck cancer treatment at the Helsinki University Hospital in 2014. The role of TORS in OPSCC treatment needs further evaluation.

RR constitutes a significant cause of mortality in OPSCC. The extent of neck treatment must take into account both treatment failures and the functional outcome. At our institute, the relative rate of ND decreased, but the rate of RR did not change. Definitive oncological treatment is more often being delivered and evaluation of the treatment response relies on PET-CT, which is performed at three months after treatment completion. However, this imaging procedure produces false-positive findings, which may predispose patients to unnecessary surgery. Further studies on treatment response imaging are therefore needed. The use of BNCT will be re-initiated at the Helsinki

University Hospital, and its role in the treatment of recurrent HNSCC will require further studies.

We were able to show that TLR 5 and TLR 7 may affect the prognosis of HR-HPV-positive OPSCC patients. These findings were obtained from small cohorts, and they will need further validation in larger cohorts. We have no biological explanation for this phenomenon. We are currently evaluating whether the downstream cascades of TLRs differ between p16-positive and p16-negative OPSCC *in vitro*. Furthermore, the treatment of OPSCC cells with TLR 5 antagonists and TLR 7 agonists should be performed in order to evaluate the possible role of TLR 5 and TLR 7 signaling in tumor progression.

11. CONCLUSIONS

1. The treatment of OPSCC changed towards a more oncological approach during the study period. Survival improved in anterior-wall disease, but in lateral-wall disease the outcome remained similar compared with the previous Finnish nationwide report. In lateral-wall disease, the prognosis was better, if the patient had a p16-positive primary tumor, no neck metastasis (cNo), or was female. In anterior-wall disease the outcome was better among patients who were female, had primary tumor class T1-T2, had a p16-positive primary tumor, or had received IMRT.
2. The rate of RR was low both in the group treated with primary surgery (4.7%) and in the group receiving definitive oncological treatment (3.2%). Older age was the only clinical factor associated with regional recurrence. All RRs occurred in patients having class cN2b or higher. Five out of the seven recurrences occurred on the contralateral side of the neck. Therefore, both sides of the neck should be treated in all patients with cN+ disease.
3. HR-HPV-positive and HR-HPV-negative OPSCC showed different TLR 7, and 9 expression patterns: TLR 7 was more expressed, and TLR 9 less expressed in HR-HPV-positive tumors. In addition, TLR 7 was more expressed and TLR 5 less expressed in p16-positive tumors. The results suggest that HR-HPV-positive and HR-HPV-negative OPSCC may evoke different immunological reactions.
4. Patients having HR-HPV-positive OPSCC had worse DSS if their tumors had high TLR 5 expression or low TLR 7 expression. As most patients who carried a HR-HPV-positive OPSCC in this series were alive with no evidence of disease at the end of the follow-up, the present findings are based on a small number of events, and further validation will therefore be needed.

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