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PROGNOSTIC BIOMARKERS, TREATMENT AND FOLLOW-UP OF T1 GLOTTIC LARYNGEAL CANCER

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DOCTORAL DISSERTATION

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To my family

ABSTRACT

Laryngeal cancer is, worldwide, the 22nd most common cancer. In Finland, those diagnosed with laryngeal cancer account annually for approximately 130 patients. The most common histological subtype is squamous cell carcinoma (SCC), covering over 95% of cases. Approximately 70% of laryngeal squamous cell carcinomas (LSCC) are glottic; the other subtypes are supraglottic and subglottic. This thesis focuses on T1 glottic LSCC, in which recurrences are rarer than at advanced stages, developing in approximately 10% of cases. The early diagnosis of recurrence is essential; delay can cause major functional deficit or mortality.

Treatment of T1 glottic LSCC with transoral laser microsurgery (TLM) or radiotherapy (RT) shows a favorable outcome. This thesis study examined the treatment, follow-up, and prognosis of T1 glottic LSCC patients at five Finnish university hospitals between 2003 and 2015. The patient total was 303. The primary treatment modality (surgery vs. RT) did not show any association with improved laryngeal preservation or 5-year disease-specific survival (DSS). The majority of the 38 recurrences were detectable during a routine follow-up visit, and of those 38, 21 (55%) of the recurrences were asymptomatic, and 15 of the recurrences were detectable more than 2 years following diagnosis of the primary LSCC. Routine post-treatment follow-up of T1 glottic LSCC seems beneficial.

Furthermore, to examine the prognosis of T1a glottic LSCC in a randomized study, we recruited 56 male patients with T1a glottic LSCC. Of these 56, 31 we randomized for TLM, and 25 for RT. The prognoses in both treatment groups of T1a glottic LSCC were similar. Five-year recurrence-free survival (RFS) in patients treated with TLM was 81%, DSS 97%, and overall survival (OS) 87%. In patients treated with RT, five-year RFS was 88%, DSS 100%, and OS 92%. The laryngeal preservation rate in both treatment groups (TLM 97% vs. RT 92%) were similar. Studies comparing treatment modalities

and prognosis of T1a glottic LSCC have been mainly retrospective. This study showed similar results in a randomized study setting.

Immunological changes in the tumor microenvironment affect enormously both tumorigenesis and tumor spread. Programmed death-ligand 1 (PD-L1) is a transmembrane protein, the overexpression of which in tumor cells can lead to T cell exhaustion and immune escape. High tumor-infiltrating lymphocyte (TIL) density is associated in many solid tumors with improved prognosis. We investigated PD-L1 expression, stromal and intratumoral TIL density, and toll-like receptors (TLRs) 4 and 5 expression in T1 glottic LSCC patients treated at five Finnish university hospitals; these totaled 174. Over half the patients had positive PD-L1 expression, yet PD-L1 showed no prognostic relevance. Low stromal TIL density was associated with local recurrence and new primary tumors of the larynx. TLR4 and TLR5 expression were not connected with survival.

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that remodel the extracellular matrix. Tissue inhibitors of MMPs (TIMPs) can deactivate MMPs. MMPs play several roles in tumorigenesis, and MMP-8 has both tumor-promoting and tumor-suppressing effects. This study included 55 recurrent respiratory papillomatosis (RRP) patients and 59 with T1-T4 LSCC. Five of the RRP patients developed LSCC during follow-up. LSCC patients and RRP patients with malignant transformation had higher S-MMP-8 levels than did RRP patients without malignant transformation. High S-MMP-8 levels predicted malignant RRP transformation. In LSCC patients, an elevated level of S-TIMP-1 was associated with poorer RFS and OS.

TIIVISTELMÄ

Kurkunpäänsyöpä on 22:ksi yleisin syöpä maailmassa ja Suomessa diagnosoidaan vuosittain noin 130 kurkunpäänsyöpää. Kurkunpäänsyövän yleisin histologinen alatyyppi on levyepiteelikarsinooma ja se todetaan yli 95 %:lla potilaista. Yli 70 % kurkunpäänsyövistä sijaitsee äänihuulissa ja loput supra- ja subglottisesti. Tämä väitöskirja keskittyy erityisesti varhaisiin T1-äänihuulisyöpiin. Vaikka tautiuusiutumat eli residiivit eivät ole yhtä yleisiä T1-äänihuulisyövissä kuin suuremissa kasvaimissa, niitä todetaan seurannassa yli 10 %:lla potilaista. Residiivin varhainen tunnistaminen on tärkeää, sillä diagnoosin viivästyminen voi aiheuttaa merkittävää toiminnallista haittaa ja lisätä kuolleisuutta.

T1-äänihuulisyöpää voidaan hoitaa kirurgisesti tai sädehoidolla ja molemmilla hoitomuodoilla ennuste on erinomainen. Väitöskirjatutkimuksessa tarkasteltiin 303 T1-äänihuulisyöpäpotilaan hoitoa, seurantaa ja ennustetta viidessä suomalaisessa yliopistosairaalassa vuosina 2003-2015. Hoitomuoto (kirurgia vs. sädehoito) ei vaikuttanut kurkunpään säilymiseen tai tautikohtaiseen eloonjäämiseen viiden vuoden seurannassa. Suurin osa 38:sta residiivistä todettiin rutiinikontrollikäynnillä ja yli puolella potilaista ei ollut uusia oireita residiivin toteamishetkellä. 15 residiiviä todettiin yli 2 vuotta primäärikasvaimen diagnosoimisen jälkeen. Tulosten perusteella T1-äänihuulisyövän rutiiniseuranta vaikuttaa hyödylliseltä.

T1a-äänihuulisyövän ennustetta selvitettiin myös satunnaistetussa tutkimusasetelmassa. Tutkimukseen otettiin mukaan 56 miespotilasta, joilla oli diagnosoitu T1a-äänihuulisyöpä. 31 potilasta satunnaistettiin kirurgisesti hoidettavien ja 25 sädehoidettavien ryhmään. Ennuste oli samanlainen molemmissa hoitoryhmissä. Kirurgisesti hoidetuilla potilailla viiden vuoden uusiutumisvapaa eloonjääminen oli 81 %, tautikohtainen eloonjääminen 97 % ja kokonaiseloonjääminen 87 %. Vastaavat tulokset sädehoidetuilla potilailla olivat 88 %, 100 % ja 92 %. Hoitoryhmien välillä ei ollut

eroa kurkunpään säilymisessä (kirurgia 97 % vs. sädehoito 92 %). Aiemmin T1a-äänihuulisyövän hoitoa ja ennustetta on selvitetty pääasiassa retrospektiivisissä tutkimuksissa. Tämä tutkimus osoitti, että tulokset ovat vastaavat myös satunnaistetussa tutkimusasetelmassa.

Syövän mikroympäristössä immuunipuolustusjärjestelmän muutokset vaikuttavat syövän kehittymiseen ja leviämiseen. PD-L1 on solukalvon proteiini, jonka yli-ilmentyminen syöpäsolussa voi johtaa T-solujen uupumiseen. Kasvaimeen tunkeutuvien lymfosyyttien eli TIL-solujen korkea määrä on yhdistetty parempaan ennusteeseen useissa eri syövissä. Tutkimuksessa selvitimme PD-L1:n, TIL-solujen ja tollin kaltaisten reseptoreiden (TLR) 4 ja 5 esiintymistä 174:llä T1-äänihuulisyöpäpotilaalla, jotka oli hoidettu viidessä suomalaisessa yliopistosairaalassa. Yli puolella potilaista kasvainkudoksen PD-L1 oli positiivinen, mutta PD-L1:llä ei ollut vaikutusta taudin ennusteeseen. Matala stromaalinen TIL-solujen määrä oli yhteydessä paikallisiin residiiveihin ja kurkunpään uusiin primäärikasvaimiin. TLR4:n ja TLR5:n esiintymisellä ei todettu olevan yhteyttä ennusteeseen.

Matriksin metalloproteinaasit (MMP) ovat sinkkiriippuvaisia endopeptidaaseja, jotka hajottavat soluväliainetta. MMP:n estäjät (TIMP) voivat vähentää MMP:n aktiivisuutta. MMP:t osallistuvat monella tavalla syövän kehittymiseen ja MMP-8:lla on sekä syövän kehittymistä edistäviä että estäviä vaikutuksia. Tutkimukseen otettiin mukaan 55 toistuvaa kurkunpään papillomatoosia (RRP) sairastavaa potilasta ja 59 T1-T4-kurkunpäänsyöpäpotilasta. Viidelle RRP-potilaalle kehittyi kurkunpäänsyöpä seurannan aikana. Kurkuunpäänsyöpäpotilailla ja RRP-potilailla, joille kehittyi kurkunpäänsyöpä, todettiin korkeampi S-MMP-8-pitoisuus verrattuna RRP-potilaisiin, joille ei kehittynyt kurkunpäänsyöpää. Korkea S-MMP-8-pitoisuus ennusti myös RRP:n malignisoitumista. Lisäksi korkea S-TIMP-1-pitoisuus oli yhteydessä kurkunpäänsyöpäpotilaiden alentuneeseen uusiutumisvapaaseen eloonjäämiseen ja kokonaiseloonjäämiseen.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Pakkanen P, Ilmarinen T, Halme E, Irjala H, Koivunen P, Pukkila M, et al. T1 glottic laryngeal cancer: the role of routine follow-up visits in detecting local recurrence. Eur Arch Otorhinolaryngol. 2021;278(12):4863–9.

II Pakkanen P, Irjala H, Ilmarinen T, Halme E, Lindholm P, Mäkitie A, et al. Survival and larynx preservation in early glottic cancer: a randomized trial comparing laser surgery and radiotherapy. Int J Radiat Oncol Biol Phys. 2022;113(1):96–100.

III Pakkanen P, Ilmarinen T, Halme E, Irjala H, Koivunen P, Pukkila M, et al. Programmed death-ligand 1, toll-like receptors and tumor-infiltrating lymphocytes (TILs) – low TIL density may predict poorer long-term prognosis in T1 laryngeal cancer. Submitted.

IV Pakkanen P, Aaltonen L-M, Sorsa T, Tervahartiala T, Hagström J, Ilmarinen T. Serum matrix metalloproteinase 8 and tissue inhibitor of metalloproteinase 1: Potential markers for malignant transformation of recurrent respiratory papillomatosis and for prognosis of laryngeal cancer. Head & Neck. 2019;41(2):309–14.

The publications are referred to in the text by their Roman numerals.

ABBREVIATIONS

Bcl-2	B-cell lymphoma 2
CD	cluster of differentation
CI	clearance interval
CO_2	carbon dioxide
CPS	combined positive score
CRT	chemoradiotherapy
CT	computed tomography
DAMP	damage-associated molecular pattern
DOD	dead of disease
DSS	disease-specific survival
ELS	European Laryngological Society
Gy	Gray
HE	hematoxylin and eosin
HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus
KTP	potassium titanyl phosphate
LC	local control
LSCC	laryngeal squamous cell carcinoma
LP	larynx preservation
LRR	leucine-rich repeat
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
NA	not available
NBI	narrow-band imaging
NCCN	National Comprehensive Cancer Network
Nd:YAG	neodymium-doped yttrium aluminum garnet
NF-κB	nuclear factor-κB
OS	overall survival
PAMP	pathogen-associated molecular pattern
PD-1	programmed cell death protein 1
PD-L1	programmed death-ligand 1
PET	positron emission tomography

RFS	recurrence-free survival
RRP	recurrent respiratory papillomatosis
RT	radiotherapy
S	serum, surgery
SCC	squamous cell carcinoma
TIL	tumor-infiltrating lymphocyte
TIMP	tumor inhibitor of metalloproteinase
TIR	toll/interleukin-1 receptor
TL	total laryngectomy
TLM	transoral laser microsurgery
TLR	toll-like receptor
TMA	tissue microarray
TPS	tumor proportion score
UICC	Union for International Cancer Control
VEGF	vascular endothelial growth factor
WHO	World Health Organization

1 INTRODUCTION

Laryngeal cancer, one of the most common head and neck cancers and the 22nd most common cancer worldwide, is diagnosed in Finland at an annual rate of approximately 130 patients.[1–3] The most important risk factors are smoking and alcohol use.[4] The most common histological subtype is squamous cell carcinoma (SCC), involved in more than 95% of all cases.[5] Approximately 70% of laryngeal squamous cell carcinomas (LSCCs) are glottic, with the other subtypes being supraglottic and subglottic LSCC. Head and neck squamous cell carcinoma (HNSCC), if diagnosis is delayed or recurrence develops, can markedly affect speaking, eating, and breathing.

Treatment of T1 glottic LSCC with transoral laser microsurgery (TLM) or radiotherapy (RT) has a favorable outcome. Because of the lack of randomized studies, these results are mainly based on retrospective studies. In T1 glottic LSCC in Finland 5-year local control, laryngeal preservation, and disease-specific survival (DSS) all exceed 80-90%.[1] The treatment decisions for each patient occur in multidisciplinary tumor board meetings in each hospital. RT is preferable when general anesthesia is unsuitable, or when the location of the T1 glottic tumor is anterior, and its visibility with TLM is insufficient. More advanced LSCCs are treated with surgery, RT, chemoradiotherapy (CRT), or combined treatment. In more advanced LSCCs and in recurrences, what is often required is total laryngectomy or laryngopharyngectomy.

Recurrences in T1 glottic LSCC are more unusual than at advanced stages, developing in approximately 10% of case, but early diagnosis of recurrence is crucial, as it can cause great functional deficit or mortality.[1] Recurrences in HNSCC are usually symptomatic, and diagnosis timing depends on the appearance of new symptoms.[6,7] The oncological outcome and cost-effectiveness of follow-up protocols should also be considered.

Observations of immunological and inflammatory changes in the tumor microenvironment have been plentiful in recent years, with two PD-1/PD-L1 inhibitors (pembrolizumab and nivolumab) approved for treatment of recurrent or metastatic HNSCC.[8–10] Despite the many studies concerning the role of various biomarkers, no biomarkers predicting the prognosis of LSCC are in clinical use. Programmed death-ligand 1 (PD-L1) is a transmembrane protein the overexpression of which can lead to T cell exhaustion and immune escape.[11] High tumor-inifiltrating lymphocyte (TIL) density is associated with improved prognosis in many solid tumors.[12]

The purposes of the current studies were to evaluate in T1 glottic cancer the oncological outcome and the effectiveness of follow-up. Our aim was also to compare treatment modalities (TLM and RT) in T1 glottic LSCC in a randomized and retrospective study design. This study investigated the prognostic role in T1 glottic LSCC of PD-L1, TILs, and toll-like receptors (TLRs). Additionally, S-MMP-8 and S-TIMP-1 underwent investigation regarding the malignant transformation of recurrent respiratory papillomatosis (RRP) and patient survival in early and advanced LSCC.

2 REVIEW OF THE LITERATURE

2.1 LARYNGEAL CANCER

2.1.1 EPIDEMIOLOGY

Laryngeal cancer is the 22nd most common cancer worldwide comprising 1.0% of all new cancer cases and 1.0% of all cancer deaths.[3] In Finland, approximately 130 new laryngeal cancers are diagnosed yearly. The incidence of laryngeal cancer in men has declined since the 1970s since smoking has reduced.[2] In women, laryngeal cancer is rarer and the incidence has remained stable. The age-adjusted incidence was 0.81 per 100 000 person-year in 2019 in women and 4.24 in men. Five-year overall survival (OS) has remained at 60%.[2] Five-year DSS in laryngeal cancer patients in Finland is 80%.[1] The prognosis of LSCC has slowly improved during the last decades.[2]

2.1.2 RISK FACTORS

Smoking and alcohol consumption are the most important risk factors for laryngeal cancer, both raising the risk dose-dependently.[4] In smokers, the relative risk for LSCC, compared to that of non-smokers, is 9.1.[13] Smoking cessation reduces the risk of LSCC by 60% after 10–15 years since cessation.[14] Other potential risk factors are environmental and occupational factors, asbestos, and advanced age.[5,15,16] High-risk human papillomavirus (HPV) is associated with oropharyngeal SCC but does not play a major role in laryngeal cancer.[17]

2.1.3 PREMALIGNANT LESIONS

Chronic laryngitis is a long-term inflammatory process in the larynx.[18] Several microbes (bacteria, viruses, fungi), laryngopharyngeal reflux, allergies, and systemic inflammatory diseases are potential causes.[19] Typical symptoms of chronic laryngitis include dysphonia, throat pain, throat clearing, cough, and globus.[18] Chronic inflammation can predispose to malignant transformation, and for instance reflux disease may be associated with LSCC.[16,20]

Laryngeal dysplasia is a precancerous condition defined as a morphologic spectrum of architectural and cytologic changes in the epithelium which can lead to laryngeal cancer.[21] In the larynx, dysplasia typically presents in the vocal cords, and hoarseness is one common symptom.[22] In laryngeal dysplasia, leukoplakia, erythroleukoplakia, hyperkeratotic and exophytic lesions are possible clinical manifestations.[22] Dysplasia diagnosis is based on biopsy but narrow-band imaging (NBI) can distinguish dysplasia from non-dysplastic lesions in an in-office setting. Positive predictive value for laryngeal dysplasia is 50% with white-light imaging and 83% with NBI; the corresponding numbers for negative predictive value are 64% and 100%.[23] The classification of laryngeal dysplasia has evolved throughout the years but the World Health Organization (WHO) Classification of Head and Neck Tumors 4th Edition divided laryngeal dysplasia in two groups: low-grade and high-grade dysplasia. Accordingly, carcinoma in situ can be separated from high-grade dysplasia.[24–26] The malignant transformation rates of laryngeal dysplasia vary widely between studies, beacuse grading, treatment, and follow-up strategies differ.[25] According to one systematic review, LSCC develops in mild dysplasia in 0%-41.7%, in moderate dysplasia 0%-48%, in severe dysplasia 14.3%-44.4%, and in carcinoma in situ, 11.1%-75% of patients.[27] In the nationwide Danish study, the overall malignant transformation rate of laryngeal dysplasia was 18% in their 10-year follow-up including 965 patients.[28]

RRP can also precede LSCC. In RRP, typically low-risk HPV types 6 and 11 cause recurrent benign, exophytic, epithelial tumors. RRP

may be juvenile-onset or adult-onset. Dysphonia is the most common symptom of RRP, but RRP can also lead to airway obstruction.[29–31] A nine-valent HPV vaccine (Gardasil, Merck) might prove useful in RRP prevention. In RRP patients, HPV vaccination may reduce papilloma regrowth after debridement and lengthen the time between surgical procedures.[29] The standard treatment of RRP is the surgical removal of papilloma tissue to maintain acceptable voice quality and an open airway, and patients may need several procedures during their lifetime. Local injections of the antiviral agent cidofovir served as adjuvant therapy. Lately, scientific interest has been focused on bevacizumab, a monoclonal antibody to vascular endothelial growth factor (VEGF).[29,31]

In earlier studies, malignant transformation has occurred in 0%-23% of the RRP cases.[32–44] In one Finnish study, the malignant transformation rate was 2.8%.[36] Classically, HPV11 has been described as more aggressive than HPV6, considering the malignant transformation. However, in the studies by Omland and Gluvajic et al., malignant transformation was more general in RRP patients with adult-onset and HPV-negative status.[34,40] Dysplasia is general among RRP patients, and 0%-55% of RRP patients present with moderate or high dysplasia. RRP patients' dysplasia rarely develops to malignancy, however.[37]

2.1.4 HISTOLOGY AND GRADING

According to the WHO Histological Classification, laryngeal cancer is categorized as malignant surface epithelial tumors (conventional SCC, verrucous SCC, basaloid SCC, papillary SCC, spindle cell SCC, adenosquamous carcinoma lymphoepithelial carcinoma), neuroendocrine carcinoma, adenoid cystic carcinoma, chondrosarcoma, and hematolymphoid malignancies.[45–47] SCC is the most common histology, covering over 95% of cases.[5] LSCC tumors are typically categorized in three grades (well, moderately, and poorly differentiated) according to their similarity to normal squamous epithelium.[26]

Table 1. Clinical TNM classification of laryngeal cancer (UICC 8th Edition).[48]

T – Primary Tumor	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ
Supraglottis	
T1	Tumour limited to one subsite of supraglottis with normal vocal cord mobility
T2	Tumour invades mucosa of more than one adjacent subsite of supraglottis or glottis or region outside the supraglottis (e.g., mucosa of base of tongue, vallecula, medial wall of piriform sinus) without fixation of the larynx
T3	Tumour limited to larynx with vocal cord fixation and/or invades any of the following: postcricoid area, pre-epiglottic space, paraglottic space, and/or inner cortex of thyroid cartilage
T4a	Tumour invades through the thyroid cartilage and/or invades tissues beyond the larynx, e.g. trachea, soft tissues of neck including deep/extrinsic muscle of tongue (genioglossus, hyoglossus, palatoglossus, and styloglossus), strap muscles, thyroid, or oesophagus
T4b	Tumour invades prevertebral space, encases carotid artery, or mediastinal structures
Glottis	
T1	Tumour limited to vocal cord(s) (may involve anterior or posterior commissure) with normal mobility T1a Tumour limited to one vocal cord T1b Tumour involves both vocal cords
T2	Tumour extends to supraglottis and/or subglottis, and/or with impaired vocal cord mobility
T3	Tumour limited to larynx with vocal cord fixation and/or invades paraglottic space, and/or inner cortex of the thyroid cartilage

T4a	Tumour invades through the outer cortex of the thyroid cartilage, and/or invades tissues beyond the larynx, e.g. trachea, soft tissues of neck including deep/extrinsic muscle of tongue (genioglossus, hyoglossus, palatoglossus, and styloglossus), strap muscles, thyroid, oesophagus
T4b	Tumour invades prevertebral space, encases carotid artery, or mediastinal structures
Subglottis	
T1	Tumour limited to subglottis
T2	Tumour extends to vocal cord(s) with normal or impaired mobility
T3	Tumour limited to larynx with vocal cord fixation
T4a	Tumour invades cricoid or thyroid cartilage and/or invades tissues beyond the larynx, e.g., trachea, soft tissues of neck including deep/extrinsic muscle of tongue (genioglossus, hyoglossus, palatoglossus and styloglossus), strap muscles, thyroid and oesophagus
T4b	Tumour invades prevertebral space, encases carotid artery, or mediastinal structures
N – Regional Lymph Nodes	
N1	Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension without extranodal extension
N2	Metastasis described as: N2a Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension without extranodal extension N2b Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension, without extranodal extension N2c Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension, without extranodal extension
N3a	Metastasis in a lymph node more than 6 cm in greatest dimension without extranodal extension

N3b	Metastasis in a single or multiple lymph nodes with clinical extranodal extension		
M – Distant Metastasis			
M0	No distant metastasis		
M1	Distant metastasis		
Stage			
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
	T1, T2, T3	N1	M0
Stage IVA	T4a	N0, N1	M0
	T1, T2, T3, T4a	N2	M0
Stage IVB	T4b	Any N	M0
	Any T	N3	M0
Stage IVC	Any T	Any N	M1

2.1.5 CLASSIFICATION

In TNM classification by the Union for International Cancer Control (UICC) tumor stage is described by tumor, lymph node metastasis, and distant metastasis as in Table 1. The larynx is divided into three subsites: supraglottis, glottis, and subglottis.[48] The supraglottis includes the epiglottis, arytenoid, false cords, and aryepiglottic folds. The glottis includes the vocal cords, and the subglottis begins 1 cm below the vocal cords.[5] In the Finnish study by Haapaniemi et al., the primary tumor was glottic in 70%, supraglottic in 25%, subglottic in 1%, and undefinable or transglottic in 4% of patients with laryngeal cancer.[1] According to TNM classification, T1 glottic laryngeal cancer is defined as a tumor which is limited to the vocal cord(s) with normal mobility. A tumor may involve an anterior or posterior commissure. A T1a tumor is limited to one vocal cord, and a T1b tumor involves both vocal cords (Figure 1).[48]

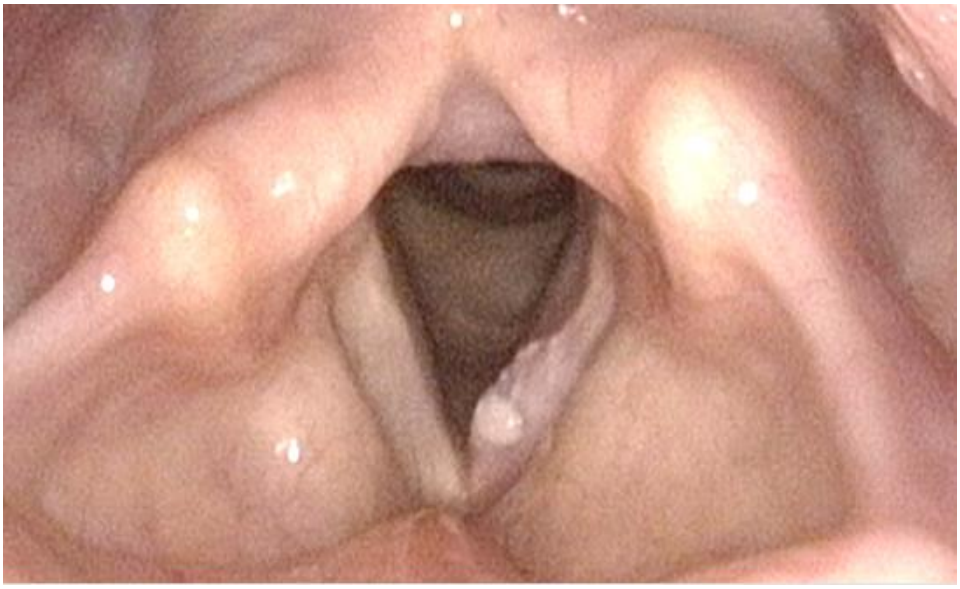


Figure 1. T1a glottic cancer. Printed with the patient's permission. Picture: Taru Ilmarinen.

2.1.6 CLINICAL PRESENTATION AND DIAGNOSTICS

The most common symptom of laryngeal cancer is hoarseness. In larger tumors, pain, dyspnea, and dysphagia may present. The symptoms can vary according to the subsite.[5,49] Since glottic tumors typically cause hoarseness, they are usually diagnosed earlier than are supraglottic and subglottic tumors.[49] In the study by Shephard et al., hoarseness had the highest individual positive predictive value (2.7%) for laryngeal cancer in primary-care patients. The positive predictive value increased when the patient had multiple symptoms simultaneously.[50]

In addition to general ear, nose, and throat examination, patients are examined with laryngoscopy using a mirror, flexible fiber-optic endoscope, rigid endoscope, or video endoscope.[49] The extension of tumor and vocal cord mobility should be considered. NBI can help in recognizing laryngeal cancer from non-malignant lesions. The diagnostic odds ratio for NBI was 87.5 compared to 13.8 for white-light imaging.[51] To confirm the diagnosis, a biopsy should be

obtained with laryngoscopy under general anesthesia or in the in-office setting with topical anesthesia.[49] Laryngoscopy under general anesthesia allows for better visualization of some laryngeal structures, including the subglottis and the lower surface of the vocal cords, and it may help to perceive if the tumor can be treated with TLM.[5] In cases with difficult exposure, a recommendable treatment option is RT.

Radiologic imaging can serve to determine the extent of laryngeal cancer and its stage. Neck metastases in stage I glottic laryngeal cancer are rare, and neck imaging can be omitted. Both computed tomography (CT) and magnetic resonance imaging (MRI) can be useful. The acquisition time of MRI is longer when motion of the larynx can cause artefacts.[52] In staging the neck, CT and MRI have an 87%-93% accuracy.[5]

2.1.7 TREATMENT

2.1.7.1 Treatment of T1 Glottic LSCC

T1 glottic LSCC be treated with RT or TLM, and single modality treatment is preferred. According to National Comprehensive Cancer Network (NCCN) guidelines, T1 glottic LSCC is treated with RT or surgery (partial laryngectomy, endoscopic or open resection).[53]

In Finland, a multidisciplinary tumor board meeting gives the treatment recommendation for each patient according to Finnish national treatment guidelines by the Finnish Head and Neck Oncology Working Group. According to these guidelines, both TLM and RT are good options in T1a glottic tumors. In larger T1a tumors extending through the whole vocal cord or in T1b tumors, the preferred treatment modality is RT. Treatment of the neck is not necessary for glottic T1aN0 tumors. In T1bN0 glottic LSCC, both RT and TLM are possible treatment modalities. TLM in T1b tumors is used rarely.[54]

Lynch introduced the transoral cordectomy technique in 1920.[55] Lillie and De Santo and Kleinsasser performed large series of transoral cordectomies with cold instruments in the 1970s.[56,57] After Steiner's publication in 1993, TLM started to spread more widely.[58] TLM can be done with a carbon dioxide (CO_2), neodymium-doped yttrium aluminum garnet (Nd:YAG) or potassium titanyl phosphate (KTP) laser.[59,60] According to the European Laryngological Society's (ELS) recommendations, TLM cordectomy types comprise nine categories depending on the degree of resection (Table 2).[61,62] The classification was updated in 2007.[62]

Table 2. The cordectomy classification by European Laryngological Society (ELS).[61,62]

Type I	Subepithelial cordectomy: resection of the epithelium
Type II	Subligamental cordectomy: resection of the epithelium, Reinke's space and vocal ligament
Type III	Transmuscular cordectomy, which proceeds through the vocalis muscle
Type IV	Total cordectomy
Type Va	Extended cordectomy, which encompasses the contralateral vocal fold and the anterior commissure
Type Vb	Extended cordectomy, which includes the arytenoid
Type Vc	Extended cordectomy, which encompasses the subglottis
Type Vd	Extended cordectomy, which includes the ventricle
Type VI	Anterior bilateral cordectomy and commissurectomy

The significance of positive surgical margins after TLM is controversial.[63] Margins can be defined as negative when a 1- to 2-mm distance between the invasive tumor front and the edge of the specimen exists.[63] Additionally, the consensus on taking resection surface biopsies is lacking. Of those patients with positive margins, 8%-51% develop recurrence during follow-up compared to a recurrence rate of 3%-23% in patients with negative margin status.[64] The positive margin status increases the risk of postoperative RT and unwanted bimodal treatment, even though a watchful waiting

policy may be practicable in many situations.[63,65] However, the association between positive margin status and the prognosis of LSCC is still unclear.

In RT of early glottic cancer, the treatment volume encompasses only the primary tumor area. The typical fractionation schedule is 66 Grays (Gy) at 2 Gy per fraction.[66] Moderate hypofractionation (2.2–2.5 Gy per fraction) compared to conventional RT may associate with higher local control.[67]

TLM and RT in T1a glottic LSCC have been compared in many retrospective studies (Table 3), with only one of those studies being prospective.[68] Their findings show that patients treated with RT and TLM have similar prognosis concerning local control, larynx preservation, DSS, and OS. Numbers of study patients range from 57 to 351, and in few studies have the number of patients in both treatment groups been comparable. In a minimum 3-year follow-up, DSS was 87%-100% in TLM and 96%-100% in RT. Larynx preservation ranged from 83%-100% in TLM and 77%-100% in RT. Local control was 69%-94% in TLM and 73%-93% in RT in a minimum of 3 years of follow-up.[68–78]

Many review articles and meta-analyses concern the treatment of early glottic cancer.[79–86] One Cochrane review compared surgery and RT in the treatment of early laryngeal cancer. In only one randomized study was open surgery compared to RT in T1-T2 glottic LSCC.[87] The Cochrane review states that there is a lack of randomized studies and their risk of bias is high.[85] In the most recent meta-analysis by Vaculik et al., including both T1a and T1b tumors and 16 studies, in both TLM and RT groups, local control was similar (85% vs. 89%). Larynx preservation and DSS on the TLM group were superior to the situation of those patients treated with RT (99% vs. 89%; 99% vs. 96%).[84] In the meta-analysis by Gioacchini et al. of T1b glottic LSCC, local control was 77% (95% confidence level (CI) 69-83%) in the patients treated with TLM and 87% (95% CI 85-89%) in those treated with RT (p=0.20). In all T1b patients, DSS was 96% (95% CI 90-98%) and OS 85% (95% CI 80-88%).[88]

Both TLM and RT of early glottic cancer may affect voice quality. In larger tumors and tumors involving the anterior commissure,

voice outcome is poorer.[89] In the meta-analysis by Du et al., no significant differences appeared in Voice Handicap Index, jitter, or shimmer between TLM and RT in T1a and Tis-T1 glottic cancer.[90] Voice outcomes in T1a glottic cancer between TLM and RT have been studied in only one randomized study. Aaltonen et al. reported similar overall voice quality in both treatment groups but the patients treated with TLM had a more breathy voice. [91]

Additionally, some factors other than survival and voice outcome affect the selection of treatment modality. The costs of RT are higher than those of TLM and total treatment time is longer with RT.[70,80,92] RT is a better option when tumor location is anterior and endoscopic exposure is difficult, or when general anesthesia cannot be considered.[89] TLM can be repeated when a recurrence develops, but in RT a curative treatment dose can be used usually only once, because the risk of adverse effects increases.[80]

Table 3. Studies comparing TLM and RT in T1a glottic LSCC. Follow-up time is a minimum 3 years. [68–78]

	No of T1a patients (N, n TLM/RT)	OS % TLM/RT	DSS % TLM/RT	LP % TLM/RT	LC % TLM/RT
Dinapoli et al. 2010	109, 61/48	No difference	87/98	NA	NA
Goor et al. 2007	89, 54/35	NA	100/100	100/97	94/90
Hirayama et al. 2002	72, 21/51	NA	100/100	100/98	91/93
Kono et al. 2016	64, 37/27	NA	100/100	100/100	92/89
Krengli et al. 2004	57, 30/27	NA	NA	83/85	96/91
Low et al. 2017	105, 53/52	86/85	100/97	100/92	69/78
Mahler et al. 2010	351, 188/163	88/81	98/97	99/93	93/89
Remmelts et al. 2013	154, 50/54	86/89	100/96	100/93	81/93
Schrijvers et al. 2009	100, 49/51	92/82	100/98	95/77, p=0.043	71/73
Sjögren et al. 2008	143, 73/70	84/81	99/96	100/86	90/79
Thurnher et al. 2007	189, 81/108	75/69	100/96	100/84	94/79

DSS, disease-specific survival; LC, local control; LP, larynx preservation; LSCC, laryngeal squamous cell carcinoma; NA, not available; OS, overall survival; RT, radiotherapy; TLM, transoral laser microsurgery.

2.1.7.2 Treatment of T2-T4 LSCC

NCCN guidelines suggest treating T2 glottic LSCC with RT or surgery, T3 glottic LSCC with CRT, RT, or surgery or both, and T4 glottic LSCC with surgery with or without postoperative RT or CRT.[53] According to Finnish treatment guidelines, T2 glottic LSCC can be treated with RT, surgery, or CRT; T2N+ and T3 glottic LSCC preferably with CRT; and T4 glottic LSCC typically with total laryngectomy and postoperative RT or CRT. [54]

For advanced glottic LSCC, possible surgery modalities are TLM, open partial laryngectomy and total laryngectomy.[59] T3 glottic LSCC is treated with CRT. However, in large T3 tumors with poor laryngeal function, total laryngectomy is preferred. Total laryngectomy is recommended treatment modality in T4 glottic LSCC.[93,94]

According to Finnish treatment guidelines, T1 supraglottic LSCC can be treated with surgery or RT and T2—T3 tumors with CRT or surgery. T4 supraglottic LSCC is treated with total laryngectomy or CRT. The neck needs to be treated with neck dissection or CRT. T1-T2 subglottic LSCC is treated with CRT and T3-T4 tumors with total laryngectomy.[54] Subglottic LSCC has a poorer prognosis than does either glottic or supraglottic LSCC.[95]

The neck is treated with CRT or neck dissection in advanced glottic LSCC. In supraglottic and subglottic LSCC, the neck needs to be treated with neck dissection or CRT.[54]

2.1.8 RECURRENCES AND FOLLOW-UP

In the Finnish study, during its 5-year follow-up, those T1-T4 LSCC patients suffering a recurrence amounted to 22%. The median time to a recurrence was 9 months, and 90% of recurrences were detected within 3 years after treatment of the primary tumor.[1] A second primary tumor is detected 15%-29% of LSCC patients during their lifetime including both HNSCC and other subsites.[96] Gao et al. reported that in 20-year follow-up, those LSCC patients diagnosed with lung cancer accounted for 19% and with other HNSCC 5%.[97]

The purposes of the follow-up are the evaluation of treatment response, early detection of recurrences and second primary tumors, observation of complications, rehabilitation, and patient support and counseling. The patient should be guided to contact healthcare if new symptoms appear.[98] Hoarseness and pain are general symptoms of recurrence. According to the ELS, during the first two years after treatment, follow-up visits at a high frequency are recommended, and follow-up continues for at least five years. The high-risk patients can benefit from prolonged follow-up.[98]

A follow-up visit includes inspection of the larynx with a mirror, flexible fiber-optic endoscope, rigid endoscope, or video endoscope according to general ENT examination. NBI can help to differentiate new neoplastic lesions from cicatrix or post-RT inflammatory changes. A biopsy under general anesthesia or in an in-office setting is necessary if a suspicion of recurrence arises. Imaging should be considered if a recurrence or a second primary tumor is possible based on new symptoms or clinical examination. Three months after RT or CRT, PET-CT allows assessment of response to treatment.[98]

HNSCC recurrences are mostly symptomatic. Pagh et al. showed that of recurrences, only 17% were asymptomatic, and the most common symptom was pain.[6] Ilmarinen et al. found that all oropharyngeal SCC recurrences were detected in patients with new symptoms.[7] Kytö et al. showed that 90% of HNSCC recurrences were found within 3 years, and asymptomatic recurrences were found during routine follow-up visits within 3 years after completion of treatment. Their conclusion was that the role of routine follow-up after three years is questionable.[99] Due to this fiding, the follow-up time of HNSCC in most of the cases in Finland is three years.[54]

The role of follow-up particularly in LSCC has been investigated. In the study by Brandstorp-Boesen et al., of 732 glottic LSCC patients, 127 (17%) had a recurrence and 103 (81%) of these recurrences were symptomatic. The patients with a symptomatic recurrence had a better post-salvage prognosis than did asymptomatic patients.[100] In the study by Ritoe et al., of 156 LSCC recurrences, 101 (65%) were symptomatic, and of 101 symptomatic

patients, 55 (54%) requested an extra visit because of new symptoms.[101]

2.1.9 CLINICOPATHOLOGICAL AND PATIENT-RELATED PROGNOSTIC FACTORS

Many prognostic clinical factors are recognizable in LSCC. Higher T stage, lymph node metastases, and advanced disease stage are connected with worse survival.[102–104] T staging acknowledges vocal cord mobility and cartilage invasion which are important prognostic factors.[48] Patients with extranodal extension in lymph node metastases have worse survival compared to the survival of patients without it.[25] Subglottic extension of the tumor and larger tumor size are also associated with worse prognosis.[104] Tumor grading plays a minor role in predicting LSCC prognosis.[26] In a meta-analysis of 4341 T1 glottic LSCC patients, when compared to the patients with anterior vocal cord involvement, those patients without anterior anterior vocal cord involvement had 12% better 5-year local control.[105]

Accordingly, multiple patient-related factors are considered to affect the LSCC prognosis. Predicting poorer survival in LSCC patients are older age (over 70 years), history of alcohol use, WHO performance status over 0, and high comorbidity.[48,102,103,106,107]

2.2 BIOMARKERS

Originally in 2000, Hanahan and Weinberg published six hallmarks of cancer which included sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastases. In 2011, they added to their proposal two new hallmarks: deregulating cellular metabolism and avoiding immune destruction. They also described two enabling characteristics: genome instability and mutation, and tumor-promoting inflammation.[108] Additionally, Hanahan presented in 2022 four new

hallmarks and enabling characteristics: unlocking phenotypic plasticity, nonmutational epigenetic reprogramming, polymorphic microbiomes, and senescent cells.[109] Molecular biomarkers observed in LSCC can be categorized by use of these hallmarks.

The tumor microenvironment is a complex entity which consists of adjacent stromal cells, immune cells, stromal cells, blood vessels, and extracellular matrix.[110] Extracellular matrix has versatile effects in regard to cancer progression. For instance, destruction of extracellular matrix is associated with tumor invasion and angiogenesis by many mechanisms.[111] The tumor microenvironment has usually evolved immunosuppressive and presents immune cell dysfunction. Cells of the tumor microenvironment deactivate T cells by many cell- and cytokine-related mechanisms. Immunotherapeutic checkpoint inhibitors try to prevent T cells drifting into exhaustion.[112,113]

In LSCC, many other well-known tumor biomarkers have also been apparent. Long non-coding RNAs are gene-expression regulators which circulate in body tissues and are detectable in serum and other tissues. In LSCC, many varying long non-coding RNAs have been associated with tumor growth, carcinogenesis and lymph node metastases.[114] Accordingly, in LSCC, many cell cycle regulators may play a role also. Ki-67 and p27 are more often expressed in advanced LSCC tumors, and they have been connected with poorer prognosis.[115] The cell cycle inhibitor p16, which plays a crucial role in oropharyngeal SCC diagnostics and treatment, is a surrogate marker for high-risk HPV. In LSCC, its importance is minor, but its expression may be higher in female, and in younger patients and non-smokers.[115] However, LSCC patients with p16-positive tumors may have better 5-year DSS than do p16-negative patients.[116,117] Apoptosis-regulatory protein B-cell lymphoma 2 (Bcl-2) and tumor-suppressor gene p53 may predict more aggressive disease and worse prognosis in LSCC patients but the results are in part controversial.[115] The results are parallel considering epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) in LSCC.[118]

2.2.1 PROGRAMMED DEATH-LIGAND 1

PD-L1 is expressed, in addition to tumor cells, also by activated T cells, dendritic cells, and monocytes. Its receptor is programmed cell-death protein 1 (PD-1), which also acts as a transmembrane protein. PD-1 can be found in activated T and B cells, monocytes, and dendritic cells.[119,120] PD-L1 overexpression is connected with T cell exhaustion on tumor cells and can lead to T cell autoinhibition and down-regulate immune responses.[121] T cells produce interferon-gamma, which leads to increased PD-L1 expression in tumor cells. In addition, hypoxia and toll-like receptor signalling can induce PD-L1 expression in tumor cells.[11] PD-L1 overexpression occurs in many malignancies. It takes part in the T-cell-specific response to tumor cells and its overexpression can lead to tumor escape from the immune system.[121–123]

The degree of PD-L1 expression can be represented by combined positive score (CPS) or tumor proportion score (TPS). CPS is calculated when the number of PD-L1-positive cells (tumor cells, lymphocytes, and macrophages) is divided by the total number of tumor cells, multiplied by 100. TPS is the number of PD-L1-positive tumor cells divided by the number of all tumor cells, multiplied by 100.[124] Many PD-L1 clones have been developed, and the percentage of stained cells can vary between the antibodies.[125] Currently, in clinical PD-L1 diagnostics, clone 22C3 is preferred, and it has developed as a selection marker for pembrolizumab. Accordingly, clone 28-8 has been developed for nivolumab, SP142 for atezolizumab, and SP263 for durvalumab.[11] PD-L1 is expressed in 50%-60% of HNSCC tumors, and its expression rate can vary between primary tumor and recurrence.[126]

Two PD-1/PD-L1 inhibitors (pembrolizumab and nivolumab) have been approved for the treatment of recurrent or metastatic HNSCC.[8–10] Positive PD-L1 expression is predictive for a response to PD-1/PD-L1 inhibitors.[9] HPV status may not affect PD-1/PD-L1 inhibitor response, but positive TIL status may improve it.[127,128] In first-line treatment, the tumor's PD-L1 status should be CPS $\geq$ 1, but in the second-line treatment, the cut-off point is TPS $\geq$ 50%.[10] In the Keynote-048 study, pembrolizumab improved OS in patients with

positive PD-L1 (CPS ≥ 1).[129] Additionally, the Keynote-040 study found that pembrolizumab improved OS when compared to the effect of standard care (8.4 vs. 6.9 years).[130] Treatment with nivolumab improved OS compared to the effect of single-agent chemotherapy in the CheckMate 141 study.[131] According to the systematic review, PD-1/PD-L1 inhibitors improved survival and reduced toxicity better than did standard care in patients with recurrent or metastatic HNSCC.[132] The WHO classification of head and neck tumors 5th Edition was published in 2022 adding the topic of checkpoint inhibitors.[47]

PD-L1's prognostic significance in HNSCC is unclear, and study results are controversial.[9] Jia et al. found that higher PD-L1 expression is associated with higher OS in nasopharyngeal SCC and higher recurrence-free survival (RFS) in LSCC.[133] Conversely, in the meta-analysis by Li et al. and Yang et al. PD-L1 was not connected with OS, RFS, or DSS in HNSCC.[134,135] Wotman et al. found no association between PD-L1 expression and the prognosis of nasopharyngeal SCC.[136] A positive correlation between PD-L1 expression and TILs has, however, been recognized.[9]

The role of PD-L1 expression is also studied in LSCC (Table 4). The studies have been retrospective, and the number of study patients ranged from 42 to 260. In those studies, clone 22C3 was generally used and CPS was reported. Follow-up time was 3 to 5 years. High PD-L1 expression was associated with better RFS and DSS in stage I-IV LSCC, and the results were observable with variable PD-L1 clones.[137–142] Accordingly, positive PD-L1 status is associated with better RFS in those LSCC patients treated with postoperative RT.[143]

Table 4. PD-L1 expression and prognosis in LSCC.[137–142]

	Antibody	No of LSCC patients	Stage	Follow-up time	PD-L1 (CPS <1/≥1)	Survival
Alessandrini et al. 2020	22C3, Dako	70	I-IV	54.5 months (median)	44/26	RFS (+)
Batur et al. 2020	22C3, Dako	52	II-IVB	41 months (median)	35/17	OS (0)
Birtalan et al. 2018	28–8, Dako	42	NA	NA	NA	DSS (+)
Franz et al. 2021	22C3, Dako	60	I-IV	58.0 months (median)	36/24	RFS (+)
Vassilakopoulou et al. 2016	Non-commercial	260	I-IV	75 months	NA	RFS (+) OS (0)
Yu et al. 2019	NA	69	I-IV	36 months (mean)	NA	OS (0)

CPS, combined positive score; DSS, disease-specific survival; LSCC, laryngeal squamous cell carcinoma; NA, not available; OS, overall survival; RFS, recurrence-free survival; 0, no correlation with PD-L1 expression and survival; +, positive correlation with high PD-L1 expression and better survival

2.2.2 TUMOR-INFILTRATING LYMPHOCYTES

The activation of immune responses is recognized as one of the hallmarks of cancer.[108] High TIL density is associated with improved prognosis in many solid tumors.[12] The Immuno-Oncology

Biomarker Working Group has created guidelines for TIL evaluation.[144]

In study of the role of TILs as a prognostic biomarker in HNSCC, the de Ruiter meta-analysis found that a high cluster of differentiation 3+ (CD3+) TILs was associated with improved OS and RFS, and high CD8+ TILs with better OS, RFS, and local control in HNSCC.[145] Low intratumoral TILs associated with poor OS and DSS in nasopharyngeal SCC and oropharyngeal SCC.[146] In the other study, in oral and oropharyngeal SCC, high TILs associated with better OS.[147,148]

High TIL density predicts improved prognosis in LSCC, and high TIL density is associated with better RFS and OS. For instance, in the previous studies, CD3+, CD4+, and CD8+ expression has been associated with the prognosis of LSCC.[140,141,149–153] In the meta-analysis by Rodrigo et al., high CD8+ and CD3+/CD4+ TILs associated with better RFS and OS.[154] The role of TILs has also been studied in recurrent/persistent LSCC, showing that high TIL density is connected with improved survival.[155,156]

The combined analysis of PD-L1 and TILs may describe more precisely immune responses in the tumor microenvironment. The tumor is immunoactive when PD-L1 and TILs are high and is noninflamed when TILs are low. When TIL density is high, but PD-L1 expression is negative, T-lymphocytes may be nonactivated or exhausted. Patients with both high PD-L1 expression and TIL density may be better responders for immune-oncological treatment.[157,158]

2.2.3 TOLL-LIKE RECEPTORS 4 AND 5

TLRs are type I transmembrane receptors which consist of intracellular Toll/interleukin-1 receptor (TIR) and extracellular leucine-rich repeat (LRR). Both immune and tumor cells can express TLRs. Pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) interact with LRR domain, which leads TIR domain to bind cytoplasmic proteins and activate several downstream networks — such as nuclear factor-

κB (NF-κB).[159–161] TLRs can be divided into extracellular TLRs (TLR1, TLR2, TLR5, TLR6, and TLR10) and intracellular TLRs (TLR3, TLR7, TLR8, and TLR9). TLR4 is expressed by both plasma membrane and intracellular compartments.[162] Different TLR agonists and antagonists have been studied in the treatment of cancer.[162]

NF-κB plays an important role in the tumor microenvironment and NF-κB activation is a part of neoplastic processes. NF-κB has both anti-tumoral and pro-tumoral impacts depending on which immune type cell is represented. Additionally, NF-κB induces directly PD-L1 gene transcription.[163,164]

The role of TLR4 and TLR5 expression in HNSCC is under study. TLR4 may predict RRP malignant transformation and its cytoplasmic expression is higher in larger LSCC tumors.[36] Both TLR4 and TLR5 are expressed in LSCC cell lines.[165] Additionally, TLR4 expression is associated with more aggressive LSCC types.[166] TLR4 and TLR5 have also been studied in oral and oropharyngeal SCC. TLR4 expression is higher in primary oropharyngeal tumors than in recurrences.[167] TLR4 expression is higher in HPV negative oral SCC than in HPV positive tumors.[168] TLR5 may predict poorer RFS and DSS in HPV-positive oropharyngeal SCC.[169] TLR5 expression is higher in oral SCC than in cutaneous SCC.[170]

2.2.4 MATRIX METALLOPROTEINASE-8 AND TISSUE INHIBITOR OF METALLOPROTEINASE-1

Matirix metalloproteinases (MMPs) are zinc-dependent endopeptidases which participate in extracellular matrix remodeling. Tissue inhibitors of metalloproteinases (TIMPs) can reduce the MMP proteolytic activity. MMP-8 degrades fibrillar collagen, an essential part of bones and ligaments. MMPs play several roles in tumorigenesis including the modification of signaling pathways and the regulation of cytokines involved in immune responses.[171] MMP-8 has both tumor-progressive and tumor-suppressive effects.[172]

In HNSCC, high TIMP-1 is associated with poorer prognosis.[173–175] Additionally, S-MMP-8 and S-TIMP-1 are higher in oropharyngeal SCC than it is in benign tonsillar disease.[176] In tongue SCC, high MMP-8 expression predicts better OS.[177] In LSCC, TIMP-1 expression may be higher in patients without lymph node metastases.[178,179] However, Ma et al. showed that TIMP-1 expression to be associated with poorer OS in LSCC.[180]

3 AIMS OF THE STUDY

The aims of the study were to observe the follow-up, treatment outcome, and survival in T1 glottic cancer. Moreover, the prognostic biomarkers in LSCC and RRP, a potential premalignant laryngeal lesion, were also in focus.

The specific aims of each study were the following:

1. To investigate the treatment outcome of T1 glottic LSCC and the role of routine follow-up visits in recurrence detection.

2. To examine long-term treatment outcome, larynx preservation, and survival of T1a glottic LSCC in a randomized study design.

3. To study the prognostic value of PD-L1, TILs, and TLRs in T1 glottic LSCC in a multicenter study, and to compile a national comprehensive series of T1 glottic LSCC samples from all Finnish university hospital biobanks.

4. To analyze whether S-MMP-8 and S-TIMP-1 are associated with malignant transformation of RRP, and with LSCC prognosis.

4 MATERIALS AND METHODS

4.1 STUDY I

Study I included 303 patients with T1 glottic LSCC, treated between 2003 and 2015 and treated at five Finnish university hospitals (Helsinki, Turku, Tampere, Oulu, Kuopio). Data from patients came from the Finnish Cancer Registry, with detailed patient information was collected from medical records in each university hospital. Statistics Finland provided data on the dates of death. The mean age of patients at the time of LSCC diagnosis was 67 years (range 29-93).

All T1 glottic LSCC patients were treated with curative intent with surgery or RT. The surgical method was mainly TLM, but one patient was treated with open surgery. RT was conventional external beam RT or intensity-modulated RT and the typical radiation dose was 66 Gy in 2-Gy fractions.

Patients had follow-up visits every 3-6 months for the first 2 years and then every 6-12 months until 5 years after treatment. In the clinical examination, an indirect laryngoscopy mirror, a fiber-optic endoscope, or a video endoscope were used. The minimum follow-up time was 3 years for 263 (87%) patients, and 205 (68%) patients had a minimum follow-up of 5 years, or until death.

LSCC in biopsy less than 6 months after treatment was defined as a residual, and after 6 months as a recurrence. LSCC tumors over 5 years after primary tumor were defined as recurrences, not new primary tumors of the larynx.

4.2 STUDY II

Study II was a sequel to the randomized voice-quality study by Aaltonen et al. In the original publication, 60 patients with stage T1aN0M0 glottic LSCC were randomly assigned to TLM and RT groups. Of these 60 patients 56 were included in the analyses. They

were treated at the three largest Finnish university hospitals (Helsinki, Turku, and Tampere) between June 1998 and October 2008. The median age of patients at the time of LSCC diagnosis was 65. Patients were randomized to treatment groups at a 1:1 ratio by means of a computer program. The treatment started within 6 weeks after diagnosis. TLM took place under in general anesthesia with CO_2 laser performed by seven experienced surgeons. RT was performed with a linear accelerator, and radiation dose was 66 Gy in 2 Gy fractions over 6.5 weeks.[91]

In Study II, medical records in each university hospital were considered for data on LSCC recurrences, total laryngectomies, and second primary tumors. Statistics Finland provided the dates of death. LSCC tumors over 5 years after diagnosis of primary T1a tumor and new primary tumors in the other areas of the head and neck or in the lungs lungs we defined as second primary tumors. Of all these patients 95% had a minimum follow-up of 5 years, or until death. In surviving patients, the median follow-up time was 6.6 years (range 3.9-17.4 years).

4.3 STUDY III

Study III included 174 T1 glottic LSCC patients who were treated between 2003 and 2013 at five Finnish university hospitals and had representative paraffin-embedded tissue blocks available for analyses. Medical records were considered in each university hospital. In Study III, LSCC tumors over 5 years after the primary tumor were defined as second primary tumors. The median age of patients at the time of LSCC diagnosis was 67 years. The minimum follow-up time was 3 years in 163 (93%) patients, or until death.

Each university hospital's biobank made tissue microarray (TMA) blocks from primary tumors. Study pathologists evaluated hematoxylin- and eosin (HE) -stained slides. From each tumor, 1-2 mm cores were removed and each was embedded in a paraffin block. TMA blocks were stained with PD-L1 antibody (clone SP142, Ventana Medical Systems, Inc., Tucson, AZ, USA, 7 ug/ml dilution).

One TMA block was stained also with PD-L1 clone 22C3 (IHC PharmDx, Dako, Carpinteria, CA, USA) as a control. Clone 22C3 is now used for diagnostics in Helsinki University Hospital. This TMA block contained 25 patients' samples (of 174, 14%).

PD-L1 expression was analyzed with a CPS algorithm. CPS is calculated when the number of PD-L1 positive cells (tumor cells, lymphocytes, macrophages) is divided by the total number of tumor cells, multiplied by 100. Membraneous immunostaining in tumor cells was considered positive. PD-L1 expression was classified as negative when CPS was under one, low when CPS was 1%-20%, and high when 21%-100%. The samples were graded by two researchers (P.P., J.H.) blinded to clinical data.

Included in TLR analyses was a subset of 109 patients treated in Helsinki and Tampere University Hospitals. For immunohistochemistry, the antibodies were TLR-4 (1:50, H-80; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and TLR-5 (1:200, IMG-664A; Biosite, Täby, Sweden). After calculation of the number of positive tumor cells, TLR expression was classified as negative with no positivity, low with 30% or less, moderate with 31-70%, and high at more than 70%. The samples were scored by two researchers (J.H., P.P.).

Eighty tissue blocks from Helsinki Biobank were further administered for TIL analyses. In TIL analyses, we used whole sections, and in a majority of cases they were HE stained. TILs we evaluated from both intratumoral and stromal regions. TIL density was assessed as the percentage of the area occupied by infiltrating lymphocytes, as described earlier.[144] When several different cutoff points for high and low stromal and intratumoral TILs were calculated, the best prognostic value for stromal TILs was at 10% (low 0-10%, high 11%-100%) and for intratumoral TILs at 1% (low 0-1%, high 2%-100%). Two researchers (P.P., A.A.) analyzed stromal and intratumoral TILs, and a senior pathologist (J.H.) verified the cases with discrepancies.

4.4 STUDY IV

In Study IV were 55 patients with RRP and 59 patients with stage I-IV LSCC without previous RRP. RRP patients were 40 years or older and had provided a serum sample between 1996 and 2004 to the Helsinki University Hospital Head and Neck Tumor Bank. The Finnish Cancer Registry provided data on all malignancies diagnosed before the year 2012. Accordingly, between January 2012 and October 2017, 29% (16 of 55) patients had been monitored, but no new LSCC tumors were diagnosed.

Included were 59 LSCC patients without preexisting RRP and had donated serum samples for the Helsinki University Hospital Head and Neck Tumor Bank at the time of LSCC diagnosis. LSCC patients were treated at Helsinki University Hospital between 2005 and 2009, with LSCC patients with distant metastases excluded. Statistics Finland provided data on dates of death. T1 LSCC patients were treated with TLM or RT. Patients with T2-T3 tumors were treated with surgery, RT, or CRT or with a combination of surgery and RT or CRT. Patients with T4 tumors were treated with total laryngectomy combined with RT or CRT or with CRT only. All patients were treated with curative intent. RT was conventional external-beam RT or intensity-modulated RT. Chemotherapeutic regimens were platinum-based agents given concomitantly with RT.

First, serum samples were centrifuged. The supernatants were stored at −70°C. MMP-8 concentrations ascertained by time-resolved immunofluorometric assay served in determining S-MMP-8 concentrations, as described by Hemmilä et al. and Tuomainen et al.[181,182] To determine TIMP-1 concentrations, we used commercially available enzyme-linked immune sorbent assay (Amersham Biosciences UK Ltd, Buckinghamshire, United Kingdom). The detection limit for MMP-8 was 0.8 ng/mL and for TIMP-1 1.25 ng/mL. For MMP-8/TIMP-1 molar ratios, the units were converted to moles per liter.

4.5 STATISTICAL ANALYSES

IBM SPSS Statistics (version 20.0, 24.0 and 27.0, Armonk, NY, USA) served for statistical analyses. Chi-square and Fisher's tests allowed study of connections between categorical variables and the t-test and Mann-Whitney-U test served for continuous variables. Survival was analyzed by the Kaplan-Meier method and log-rank test. In Study IV, patients with LSCC were divided into three groups according to serum concentrations (highest quintile, 3 middle quintiles, and lowest quintile). Cox regression served for analysis of associations between log10 transformed serum values, adjusted for age, sex, stage (I-II vs III-IV), and survival. A statistically significant p-value was set at 0.05.

Local control was defined as duration from diagnosis of primary LSCC tumor to the first documented local recurrence. RFS was defined as the duration from diagnosis to the first recurrence (local, regional, or distant). DSS was defined as duration from diagnosis to death from LSCC. OS was defined as duration from diagnosis to death from any cause.

4.6 ETHICAL CONSIDERATIONS

The Ethics Committee of Surgery approved Studies I-IV. Their institutional study permissions were granted by each university hospital. In Study IV, all patients provided their informed written consent, and in Study II patients had given written consent before study participating in the original study. In Studies I and III, no informed consent was required rule concerning patients in a retrospective study design, according to the Finnish law. The studies followed the guidelines of the Declaration of Helsinki.

5 RESULTS

5.1 STUDY I

Patient characteristics in Studies I-III appear in Table 5. In Study I, of 303 patients, 163 (54%) were treated with surgery (162 with TLM and one with open surgery) and 140 (46%) with RT. The portion of the patients treated with surgery varied between study years 2003 and 2015, but the difference was not statistically significant (range 36-77%, p=0.263, Figure 2). T1a glottic LSCC patients had surgery more often than did those patients with T1b LSCC (58% vs. 17%, p<0.001). Involvement of the anterior third of the vocal cord did not relate to primary treatment method in T1a glottic LSCC.

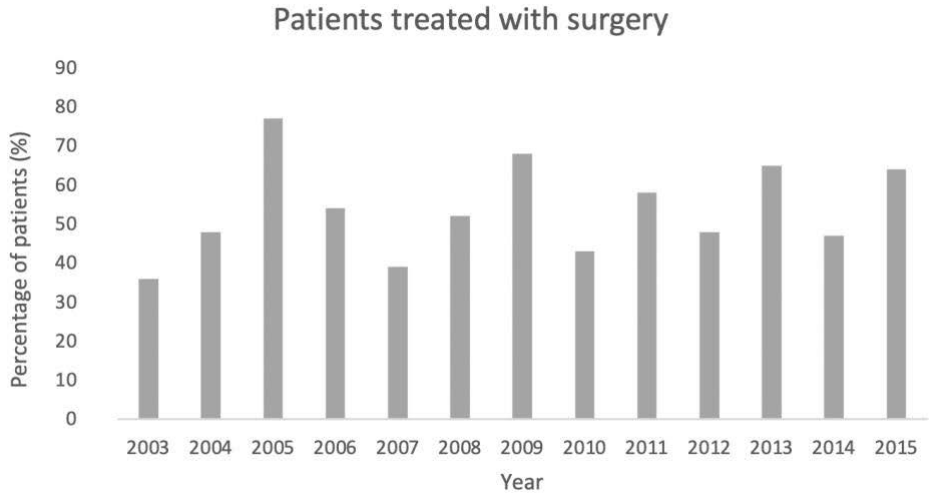


Figure 2. Percentage of patients treated with surgery in Study I.

T1a patients treated with TLM showed significantly more local recurrences than did patients treated with RT: 25 (16%) vs. 6 (5%), p=0.006. Of 25, 20 (80%) local recurrences in patients treated with TLM were diagnosed within 5 years after their primary tumor. T1a patients treated with RT had higher 5-year local control than did patients treated with TLM (97% vs. 87%, p=0.020).

Among the 158 T1a LSCC patients treated with surgery, resection surface margins were reported in 86 (54%), and of these 86, 71 (83%) had negative resection margins; 7 of them (10%) had local recurrence during follow-up. Of 86 T1a patients treated with surgery, 15 (17%) had the status of positive resection margin. Of these 15, 7 had a re-resection, and 8 were followed up with a wait-and-see protocol. Of the 7 patients with re-resection, 2 (29%), and of the 8 with follow-up, 3 (38%) had a local recurrence. The local recurrence rate was higher in T1a LSCC patients with positive margin status than in those with negative resection margins (33% vs. 10%, p=0.031). Neither positive nor negative margins were associated with DSS in T1a LSCC patients treated with surgery (p=0.064).

Of all 303 (13%) T1 glottic LSCC patients, 38 (13%) had a recurrence during follow-up. For the patients with local recurrence, see Table 6. Of the recurrences, 35 (92%) were local and 3 (8%) regional or distant. Local recurrences were diagnosed 1.7 years after the primary tumor (median, range 0.6-8.9 years). Of 35 local recurrences, 10 (29%) local recurrences were diagnosed over 3 years, and 7 (20%) over 5 years after the primary LSCC tumor. All patients' 5-year RFS was 91%, T1a patients' 91% and T1b patients' 86%. All patients' 3-year RFS was 92%, T1a patients' 92% and T1b patients' 90%. T stage did not relate to 5-year RFS (p=0.831) in a log-rank test.

Table 5. Patient characteristics in Studies I-III.

	Study I (N=303)	**Study II (N=56)**	**Study III (N=174)**
Sex, n (%)			
Male	263 (87)	56 (100)	146 (84)
Female	40 (13)	0 (0)	28 (16)
Smoking history, n (%)			
Yes	256 (85)	48 (86)	147 (84)
No	33 (11)	4 (7)	19 (11)
NA	14 (5)	4 (7)	8 (5)
Earlier dysplasia*, n (%)			
Yes	32 (11)	6 (11)	12 (7)
No	240 (79)	39 (70)	143 (82)
NA	31 (10)	11 (20)	19 (11)
T stage, n (%)			
T1a	274 (90)	56 (100)	156 (90)
T1b	29 (10)	0 (0)	18 (10)
N stage, n (%)			
0	302 (100)	56 (100)	174 (100)
1	1 (0)	0 (0)	0 (0)
Primary treatment, n (%)			
Surgery	163 (54)	31 (55)	100 (57)
RT	140 (46)	25 (45)	74 (43)
Recurrence, n (%)			
Local	35 (12)	8 (14)	20 (11)
Regional or distant	3 (1)	1 (2)	1 (1)
No	265 (87)	47 (84)	153 (88)
Second primary tumor, n (%)			
Yes	20 (7)	10 (18)	20 (11)

No	283 (93)	46 (82)	154 ()
Died of LSCC, n (%)			
Yes	8 (3)	2 (4)	4 (2)
No	295 (97)	54 (96)	170 (98)

LSCC, laryngeal squamous cell carcinoma; NA, not available. *Presence of dysplasia confirmed in laryngeal biopsy before the diagnosis of T1 glottic cancer.

Of 38 recurrences, 26 (68%) were diagnosed on a routine follow-up visit by clinical examination. Nine patients (24%) with new symptoms booked an extra visit. Two (5%) of the recurrences were detected during examination of another disease and one (3%) was diagnosed by positron emission tomography (PET) imaging used in follow-up in one university hospital. Of 38 patients with recurrence, 21 (55%) were asymptomatic at diagnosis of recurrence. Hoarseness (in 13 patients), pain (3), and dysphagia (3) were the most common symptoms in patients with recurrence. The time from primary LSCC diagnosis to detection of local recurrence in asymptomatic patients was significantly shorter than in symptomatic patients (median 1.6 years (range 0.6–8.9 years) vs. median 2.7 years (range 0.9–8.4 years), p=0.019). The pattern of symptoms (symptomatic vs. asymptomatic) showed no connection with larynx preservation rate (60% vs. 70%, p=0.721).

Larynx preservation rate was unrelated to primary treatment method (surgery 5 of 163 (3%) vs. RT 8 of 140 (6%), p=0.395). Of the 303 patients, 20 (7%) developed a second primary tumor in follow-up, and 9 (3%) died of a second primary tumor. The primary treatment method of LSCC showed no association with second primary tumors (p=0.248). Of the same 303 patients, 8 (3%) died of LSCC. Five-year DSS was 98% in all patients, 97% in T1a LSCC patients, and 100% in T1b LSCC patients. The corresponding numbers for 5-year OS were 82%, 82%, and 76%. DSS and OS were not associated in a log-rank test either with T stage or with primary treatment method.

Table 6. Patients with local recurrences in Study I (n=35).

Patient	Sex, age (y)	T stage, primary treatment, margin status*	Time to recurrence after primary diagnosis (y)	Recurrence detected on a routine follow-up visit	New symptoms	Outcome	TL
1	M, 84	T1b, RT	2,4	Yes	No	Died of other causes	No
2	F, 48	T1a, S, positive	5,5	Yes	No	Alive w/o disease	No
3	M, 67	T1a, S, negative	0,9	Yes	No	DOD	Yes
4	F, 59	T1a, S, negative	1,5	Yes	No	Alive w/o disease	No
5	M, 66	T1a, RT	1,8	Yes	No	Alive w/o disease	Yes
6	M, 66	T1a, S, positive	1,1	Yes	No	Alive w/o disease	No
7	M, 79	T1a, S, negative	0,7	Yes	No	Alive w/o disease	No
8	M, 72	T1a, S, negative	1,0	Yes	No	Alive w/o disease	No
9	M, 65	T1a, S, NA	0,9	Yes	No	Alive w/o disease	No

10	F, 76	T1b, S, NA	0,6	Yes	No	Alive w/o disease	Yes
11	F, 78	T1a, S, NA	0,9	Yes	No	Alive w/o disease	No
12	F, 75	T1a, S, NA	1,7	Yes	No	Alive w/o disease	Yes
13	M, 54	T1a, S, NA	1,5	Yes	No	Died of other causes	No
14	F, 70	T1a, S, NA	2,1	Yes	No	Died of other causes	No
15	M, 58	T1a, RT	1,7	Yes	No	Alive w/o disease	Yes
16	M, 72	T1a, S, NA	1,6	Yes	No	Alive w/o disease	No
17	M, 84	T1a, S, NA	3,1	Yes	No	DOD	No
18	M, 59	T1a, S, positive	0,8	Yes	No	Alive w/o disease	No
19	M, 66	T1a, S, positive	2,7	Yes	Hoarseness	Alive w/o disease	No
20	M, 88	T1a, S, positive	1,4	Yes	Hoarseness	DOD	Yes
21	F, 60	T1a, S, NA	3,2	Yes	Hoarseness	Alive w/o disease	No
22	M, 56	T1a, S, negative	8,3	Yes	Hoarseness, cough	Alive w/o disease	No
23	F, 56	T1b, RT	0,9	Yes	Hoarseness	Alive w/o disease	Yes

24	M, 79	T1a, S, NA	1,6	Yes	Hoarseness	Died of other causes	No
25	M, 65	T1a, RT	8,9	No	No	Alive w/o disease	No
26	M, 52	T1b, RT	2,4	No	No	Died of other causes	Yes
27	M, 55	T1a, S, negative	8,0	No	Hoarseness	Alive w/o disease	No
28	M, 48	T1a, RT	8,2	No	Hoarseness, throat pain	Alive w/o disease	Yes
29	M, 80	T1a, S, negative	3,8	No	Hoarseness, dysphagia	Died of other causes	No
30	M, 79	T1a, S, NA	8,4	No	Hoarseness	Alive w/o disease	No
31	M, 57	T1a, S, NA	2,2	No	Hoarseness	Alive w/o disease	No
32	M, 67	T1a, S, NA	5,3	No	Hoarseness, dysphagia, odynophagia	Alive w/o disease	No
33	M, 73	T1a, S, negative	1,7	No	Hoarseness	Died of other reasons	Yes
34	F, 55	T1a, RT	1,2	No	Throat pain	Alive w/o disease	Yes

35	M, 55	T1a, RT	2,0	No	Dysphagia	DOD	Yes

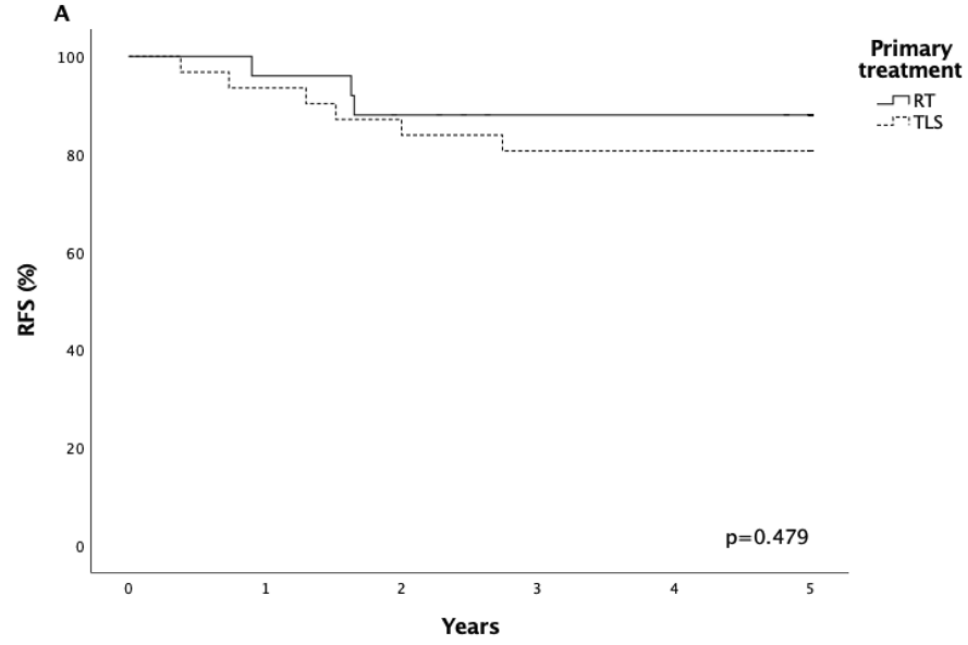
DOD, dead of disease; F, female; M, male; NA, not available; RT, radiotherapy, S, surgery; TL, total laryngectomy. *The margin status in patients treated with transoral laser microsurgery.

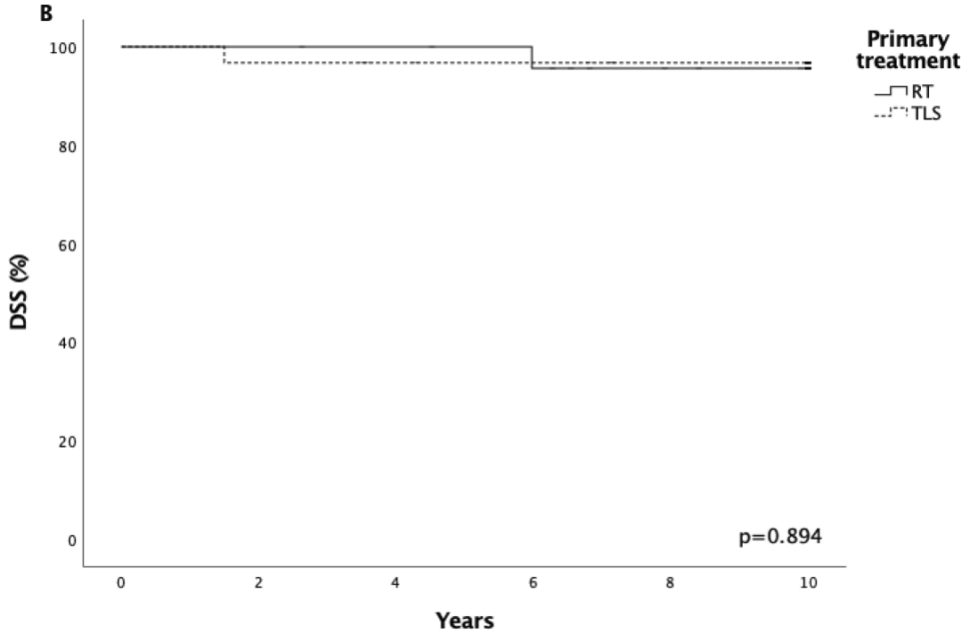
5.2 STUDY II

In Study II, of 56 T1a glottic LSCC patients, during a 5-year follow-up, 9 (16%) had a recurrence. The recurrence was diagnosed 1.5 years after the primary LSCC tumor (median, range 0.4-2.7 years). Eight of the recurrences were local and one distant. All patients' 5-year RFS was 84%, patients' treated with TLM 81%, and with RT 88% (Figure 3A). The primary treatment method was not associated with larynx preservation (p=0.575). In a 5-year follow-up, 3 (5%) patients had a total laryngectomy.

Of the 56 patients, 10 (18%) developed a second primary tumor in follow-up. The subsites were 5 in the lungs, 3 in the larynx, and 2 in the hypopharynx. The median time from LSCC diagnosis to second primary tumor was 8.5 years (range 3.8-15.4 years). Tumors of the larynx were treated with surgery (2 patients) or with RT (one). Four of the patients died of a second primary tumor.

One (2%) patient died of LSCC in 5-year follow-up, and one patient died more than 5 years after LSCC diagnosis. Five-year DSS was 98% in all patients, 97% in patients treated with TLM and 96% in patients treated with RT (10-year DSS 97%, 97%, 96%, Figure 3B). The corresponding numbers for 5-year OS were 89%, 87% and 92% (10-year OS 20%, 19%, 20%, Figure 3C). DSS and OS were not associated with primary treatment method in a log-rank test.


A
RFS (%)
100
80
60
40
20
0
0
1
2
3
4
5
Years
p=0.479
Primary treatment
RT
TLS


B
DSS (%)
100
80
60
40
20
0
0
2
4
6
8
10
Years
p=0.894
Primary treatment
RT
TLS

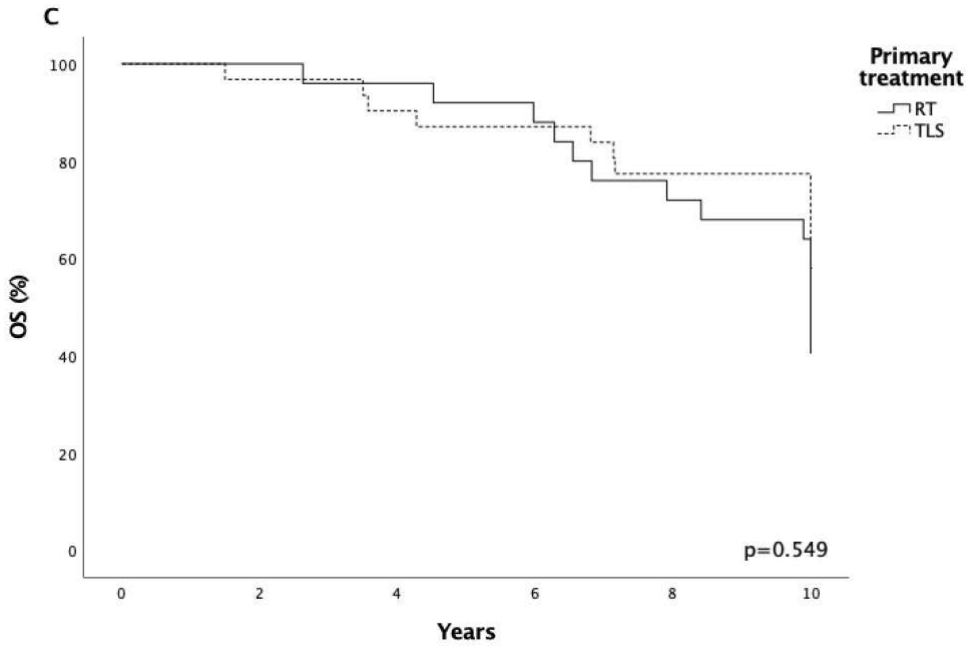


Figure 3. A) Five-year recurrence-free survival (RFS) according to primary treatment method in glottic T1a LSCC patients in Study II. B) Ten-year disease-specific survival (DSS) in glottic T1a LSCC. C) Ten-year overall survival (OS) in glottic T1a LSCC.

5.3 STUDY III

Of 174 patients, 20 (12%) had a local recurrence and one (0.6%) a distant recurrence during a 5-year follow-up; 9 (5%) patients had a total laryngectomy and 20 (11%) patients had a second primary tumor in follow-up. Five-year DSS of all patients was 98%. In total, of 174 patients, 4 (2%) patients died of LSCC.

PD-L1 analyses included these 174 patients, of whom 32 (18%) showed high, 67 (39%) low, and 75 (43%) negative PD-L1 expression. PD-L1 expression was identical with both SP142 and 22C3 clones. Local control, larynx preservation rate, DSS, and OS did not correlate to PD-L1 expression in a log-rank test. All patients who died of LSCC had low or high PD-L1 expression.

Of 109, 40 (37%) had negative, 45 (41%) low, 22 (20%) moderate, and 2 (2%) high TLR4 expression. In TLR5 analyses corresponding numbers were 2 (2%), 15 (14%), 70 (64%), and 22 (20%). Neither TLR4 nor TLR5 correlated with larynx preservation rate, 5-year OS, local control, or DSS. PD-L1 expression did not associate with TLR4 or TLR5 expression.

Of the 174 patients, 80 (46%) were included in TIL analyses, with 30 patients (38%) having high and 50 (63%) low stromal TIL density and 11 (14%) high and 69 (86%) low intratumoral TIL density. All patients with low intratumoral TIL density also had low stromal TIL density. Of 80 patients, 6 (8%) had a local recurrence in a 5-year follow-up, and all of them had low stromal and intratumoral TIL density. Of 74 patients without local recurrence, 44 (59%) had low and 30 (41%) had high stromal TIL density, and 63 (85%) had low and 11 (15%) high intratumoral TIL density. Stromal TIL density did not associate with 5-year local control statistically significantly ($p=0.052$). Intratumoral TIL density did not relate to 5-year local control. Compared to those patients with no LSCC events during follow-up, low stromal TIL density was more common in patients with either local recurrence or a new primary tumor of the larynx (detected over 5 years from diagnosis, n=5; low stromal TILs 58% vs. 91%, $p=0.047$). Stromal and intratumoral TIL density was not associated with larynx preservation rate or second primary tumors. All three (4%) of the patients included in TIL analyses who died of LSCC had low stromal and intratumoral TIL density, but their 5-year DSS did not associate with stromal or intratumoral TIL density in a log-rank test.

In combined PD-L1 and stromal TIL analyses we included 80 patients, and defined PD-L1 as positive or negative (CPS 1-100 vs. 0, Table 7). Patients with positive PD-L1 had higher stromal TIL density than did those with negative PD-L1 ($p=0.037$). Local recurrences, larynx preservation rate, or second primary tumors were not associated with combined PD-L1 and stromal TILs.

Table 7. Combined PD-L1 expression and stromal TILs (n=80).

	Low stromal TILs (0-10%, n=50)	High stromal TILs (11-100%, n=30)	p-value
PD-L1 < 1 (n=40)	30 (38%)	10 (13%)	0.037
PD-L1 1-100 (n=40)	20 (25%)	20 (25%)	

PD-L1, programmed death-ligand 1; TIL, tumor-infiltrating lymphocytes.

5.4 STUDY IV

Study IV included 55 patients with RRP and 59 patients with LSCC. Of the RRP patients 42 (76%) were male and 13 (24%) female. RRP patients' median age was 55 years (range 40-77 years). Five of 55 patients with RRP developed LSCC in follow-up. All of them were male. For patients with malignant transformation median age at the time of RRP diagnosis was 60 years (range 4-73 years) and RRP was diagnosed 7 years (3-63 years) before LSCC. Of patients with LSCC, 50 were male and 9 female. Their median age at diagnosis was 66 years (range 26-68 years). The subsite of LSCC was glottis or transglottic in 51 (86%) patients, supraglottis in 7 (12%), and subglottis in one (2%). T stage was T1 in 19 (32%) patients, T2 in 14 (24%), T3 in 15 (25%), and T4 in 11 (19%). Of 59 LSCC patients, 13 (22%) had a recurrence during follow-up, and 8 (14%) died of LSCC.

The LSCC patients without preexisting RRP had higher S-MMP-8 levels and MMP-8/TIMP-1 molar ratio than did patients with RRP (mean 35.0 vs. 116.1 ng/ml, $p<0.001$; 0.40 vs. 0.11 ng/ml, $p<0.001$). These differences were not evident when S-TIMP-1 levels were compared (141.3 vs. 153.8 ng/ml, $p=0.050$). Patients with T1 LSCC (n=19) had higher S-MMP-8 level and MMP-8/TIMP-1 molar ratio than did RRP patients (94.9 vs. 35.0 ng/ml, $p=0.002$; 0.40 vs. 0.11, $p=0.01$). The serum values are presented in Table 8.

RRP patients with malignant transformation had higher S-MMP-8 levels and MMP-8/TIMP-1 molar ratio than did other RRP patients (70.1 vs. 31.5 ng/ml, p=0.01; 0.23 vs. 0.10, p=0.009). S-TIMP-1 levels were, however, similar in both groups (129.4 vs. 142.5 ng/ml, p=0.23). Age showed no association with the malignant transformation of RRP (p=0.05).

Patients with stage III-IV LSCC had higher S-MMP-8 levels and MMP-8/TIMP-1 molar ratios than did patients with early-stage LSCC (91.7 vs. 143.1 ng/ml, p=0.01; 0.46 vs. 0.35, p=0.03). S-TIMP-1 level was not associated with stage of LSCC (I-II 150.8 vs. III-IV 157.1 ng/ml, p=0.63). Levels of S-MMP-8 and S-TIMP-1 LSCC patients without preexisting were associated with OS in log-rank test (p=0.004; p=0.04).

Log10 transformed S-TIMP-1 values were associated with RFS (0.05) and OS (p=0.02) in multivariate analysis adjusted for sex, age and stage (I-II vs. III-IV), but not associated with DSS (hazard ratio 12.0, 95% confidence interval 0.95-152.2, p=0.05. Log10 transformed S-MMP-8 level and MMP-8/TIMP-1 molar ratio did not associate with RFS, DSS, or OS.

Table 8. S-MMP-8 and S-TIMP-1 levels and S-MMP-8/TIMP-1 molar ratios in RRP and LSCC patients.

	RRP (n=55)	**LSCC (n=59)**	**RRP without malignant transformation (n=50)**	**RRP with malignant transformation (n=5)**	**LSCC stage I-II (n=31)**	**LSCC stage III-IV (n=28)**
S-MMP-8 (ng/ml), mean (range)	35.0 (7.2-115.6)	116.1 (9.1-454.7)	31.5 (7.2-82.8)	70.1 (23.5-115.6)	91.7 (9.1-454.7)	143.1 (18.7-363.2)
p value	<0.001		0.01		0.01	
S-TIMP-1 (ng/ml), mean (range)	141.3 (72.6-195.1)	153.8 (48.9-391.8)	142.5 (72.6-195.2)	129.4 (101.6-154.3)	150.8 (48.9-391.8)	157.1 (80.1-301.1)
p value	0.50		0.23		0.63	
MMP-8/TIMP-1 molar ratio, mean (range)	0.11 (0.02-0.38)	0.40 (0.03-2.71)	0.10 (0.02-0.30)	0.23 (0.08-0.38)	0.35 (0.03-2.71)	0.46 (0.05-1.23)
p value	<0.001		0.009		0.03	

LSCC, laryngeal squamous cell carcinoma; MMP-8, matrix metalloproteinase 8; RRP, recurrent respiratory papillomatosis; TIMP-1, tissue inhibitor of metalloproteinase 1.

6 DISCUSSION

6.1 TREATMENT OF T1 GLOTTIC LSCC

The prognosis of T1 and particularly of T1a glottic LSCC, according to Studies I-II, is excellent. These studies show that choice of treatment modality, TLM or RT, does not affect the oncological prognosis in a randomized or retrospective study design. The majority of T1 glottic LSCC recurrences perform without new symptoms, and the recurrences are frequently detectable on a routine follow-up visit.

To our knowledge, Study II is the first randomized study comparing the effect of TLM and RT on the prognosis of T1a glottic LSCC. Earlier, randomized studies have ended because of their poor accrual.[85] Those studies including both T1a and T1b tumors, as well as Study I, have been mainly retrospective, resulting in significant selection bias.[68–78] For instance, some studies have found inferior larynx preservation or OS in patients treated with RT compared to results for patients treated with TLM. Possibly, the RT-treated patients may have presented with larger T1a or T1b tumors with a greater risk of recurrence. These results underline the importance of our randomized study.

In Study I, T1b glottic LSCC patients underwent RT more often than did T1a patients, as expected. T stage was not associated with the prognosis of T1 glottic LSCC in Study I. In the literature, the percentage of T1b glottic LSCC patients treated with RT has varied. In Finnish series by Haapaniemi et al., 20% of T1b patients underwent surgery.[1] One meta-analysis including 52 studies showed inferior RFS in T1b glottic LSCC patients treated with TLM compared to RFS in patients treated with RT (77% vs. 87%), but between those groups, DSS and OS did not differ.[88] Voice quality in T1b patients treated with TLM is often acceptable but the patient series have been small.[183] In conclusion, the RT is a preferred

treatment method in T1b glottic LSCC but TLM may be feasible in cases with good visibility and small tumors.

Studies I-II found no association between treatment method and the oncological outcome of T1 glottic LSCC as expected: 5-year RFS, larynx preservation, 5-year DSS, and OS were comparable. T stage did not affect the prognosis of T1 glottic LSCC patients in Study I, except that those T1a patients treated with TLM had inferior 5-year local control when compared to that of the patients treated with RT. Potential errors in T staging, missing margin status, and surgeons' TLM learning curve may explain these results. Bernal-Sprekelsen et al. found no connection between surgeons' TLM experience (< 30 procedures vs. 31-60 procedures vs. > 60 procedures) and the prognosis of early laryngeal and hypopharyngeal tumors but in locally advanced tumors the difference was significant.[184] In Finland, treatment of HNSCC is centralized to tertiary hospitals to ensure sufficient expertise. Since the long-term prognosis of T1 glottic LSCC is favorable with both treatment methods, choice of treatment modality should be based on the other factors. Patient preferences, the availability of TLM and RT, the costs, and the expectations for posttreatment voice quality should all be considered. A multidisciplinary tumor board meeting is essential in opting for the best treatment modality for each patient. A randomized study by Aaltonen et al. showed that T1a glottic LSCC patients treated with TLM had a more breathy voice, and the glottal cap was wider than in patients treated with RT.[91] Accordingly, a biomarker predicting response to RT, or CRT in advanced LSCC, could prove helpful in each treatment decision. This has already been remarked upon, but no biomarkers are yet in clinical use.[185,186] More studies concering this topic are necessary in the future.

In our study, T1a glottic LSCC patients with positive margin status more often had local recurrences than did the patients with negative margins. Of 163 T1 glottic LSCC patients primarily treated with TLM, 11 (7%) had a residual tumor and required RT postoperatively, leading to unwanted bimodal treatment. Of T1a glottic LSCC patients treated with surgery and with reported margin status, 17% had positive margins. These findings are comparable

with those earlier studies. Hanna et al. reported a positive margin rate of 17.3% in T1 LSCC patients.[65] However, the prognostic significance of margin status is controversial since results are variable between the studies. Some of the studies found no association between the positive margin status and the prognosis.[63] The approach to positive margin status should be a factor considered when follow-up protocols are updated in future. In Study I, margin status was reported for only half of the patients. Even if the study evidence concerning margin status in T1 glottic LSCC remains unclear, margin status should be systematically reported in every patient treated with TLM. Further studies concerning this topic are also essential.

Study I included approximately 60% of all T1 glottic LSCC patients in Finland between 2003 and 2015. In those years, patients were also treated in non-tertiary hospitals, but since 2018 the treatment of HNSCC has become centralized in tertiary hospitals. For some patients, follow-up took place in non-tertiary hospitals when complete follow-up data was lacking. The present study, being retrospective, means that detailed data on new symptoms caused by a recurrence, on smoking, on an earlier dysplasia, or on margin status is missing for some patients.

The accrual of Study II took over 10 years, and approximately 80% of the T1a glottic LSCC patients refused to take part in the study. Because the original study guaranteed homogeneity in voice analysis and thus included only male patients, Study II, drawing from that same patient pool, included none who were female. In consequence, Study II does not represent all patients with T1a glottic LSCC — women, for instance. The oncologic outcomes of patients treated with TLM and RT were similar. Study II included only 56 patients, of whom, only 2 patients died of LSCC. To show significant differences in major endpoints requires larger series.

6.2 FOLLOW-UP OF T1 GLOTTIC LSCC

In Study I, over half the recurrences in T1 glottic LSCC patients were detectable on a routine follow-up visit, and most of these recurrences were asymptomatic. These findings differ from those in other HNSCCs, in which the majority of recurrences are detectedable when new symptoms appear.[7] In T1 glottic LSCC, patients may adjust to long-term or permanent voice changes, and this may hinder symptom-directed surveillance. Consequently, the first symptoms of recurrence may be stridor and airway obstruction. In Study I, patients with early (follow-up less than 2 years) recurrence were more often asymptomatic, and thay requested an extra visit less frequently. The follow-up protocol is typically intense during the first two years when even patients who are symptomatic may remain waiting for the next follow-up visit. In two studies observing the follow-up of LSCC, the number of symptomatic patients was substantially higher, because those studies included both early- and advanced-stage LSCCs.[100,101] In Study I, we found no connection between symptoms and larynx preservation rate. However, in the literature symptomatic LSCC recurrences may have had a better prognosis than did those that were asymptomatic.[100] The follow-up of T1 glottic LSCC patients can be assessed with endoscope and NBI in an in-office setting instead of by radiologic examinations. This reduces costs and exposure to radiation. The follow-up protocols should be considered critically to improve their utility to detect recurrences in a well-timed and cost-effective manner. Any delay in recurrence detection may worsen oncological and functional outcome. Our results show that routine follow-up seems beneficial in T1 glottic LSCC. At least, follow-up during the first 3 years is essential since many recurrences are asymptomatic.

In Studies I-II, only a minority of patients with recurrence died of it during follow-up. These findings indicate that the treatment of T1 glottic LSCC recurrences is feasible and should always be considered. Compared to other HNSCC, LSCC recurrences are more often curable, and LSCC patients' prognosis is better.[187] Systematic

review by Russo et al. proposed that salvage TLM after primary (C)RT is a valuable treatment option in selected patients.[188] In addition to detecting the recurrences, the potential purpose of follow-up from an oncological point of view is to observe possible second primary tumors. In Studies I-II, 7%-18% of patients were diagnosed with a second primary tumor during follow-up, but the definition of second primary tumor varied between studies. In neither study was the primary treatment method associated with second primary tumors, findings corresponding with those of earlier studies, in which the risk of a second primary tumor in LSCC patients ranged from 15% to 29%.[96] Smoking is a major risk factor for LSCC and lung cancer, and smoking cessation may reduce the risk of a second primary tumors.[189] Smoking cessation may improve local control of T1a glottic LSCC patients treated with RT.[190] LSCC patients should always be encouraged to cease smoking.

Early (T1-T2) and advanced (T3-T4) LSCC differ significantly, and having only one common follow-up protocol is not often the most practical solution. For instance, in advanced disease, the role of PET-CT is essential in evaluation of response to RT or CRT treatment. The approach to recurrences also differs between early and advanced LSCC. In early LSCC, in treatment of recurrences the main objective is laryngeal preservation. In advanced LSCC, when a recurrence develops, total laryngectomy is often the sole option. Early and advanced LSCC should thus also be viewed as separate entities, and the results of Studies I-II help to develop the follow-up of T1 glottic LSCC.

LSCCs occurring more than 5 years after diagnosis of the primary tumor we considered to be new primary tumors of the larynx in Study III. This is in contast with Study I, in which such tumors we defined as late local recurrences. In Study II, new tumors of the larynx over 5 years after primary LSCC diagnosis we defined as second primary tumors. Our varying definitions between Studies I, II, and III indicate wider challenges in this area, and the lack of any proper definitions for a local recurrence and a new primary tumor. One systematic literature review suggests that a local recurrence of HNSCC is one that is detected within 3 years after treatment of the

primary tumor and that is located within 3 cm of the primary tumor.[191] Additionally, the prognosis of local recurrences is generally inferior to that of second primary tumors, since the latter can be treated more often with curative intention.

6.3 BIOMARKERS IN T1 GLOTTIC LSCC

Cancer progression is a complex process, and in recent years, immunological and inflammatory changes in the tumor microenvironment have been a popular topic of HNSCC cancer study. In HNSCC, two PD-1/PD-L1 inhibitors (pembrolizumab and nivolumab) have been approved for treatment of recurrent or metastatic disease.[8–10] Study III showed that low stromal TIL density was associated with local recurrences and new primary tumors of the larynx in T1 glottic LSCC patients. We found that PD-L1 and TLR expression played no prognostic role.

In Study III, PD-L1 was positive in over half of the T1 glottic LSCC patients, but PD-L1 did not connect with its prognosis. In earlier studies, high PD-L1 expression has been associated with improved prognosis in stage I-IV LSCC.[137–142] In advanced-stage LSCC, recurrences and deaths are more frequent, and the immune system can act differently from the situation in early-stage LSCC, facts that can explain these findings. High PD-L1 is considered the best predictive biomarker for response to PD-1/PD-L1 inhibitors.[10] Over 10% of patients with T1 glottic LSCC develop a recurrence during follow-up. Although metastatic recurrences in T1 glottic LSCC are rare, such patients are potential candidates for PD-1/PD-L1 immunotherapy. This indicates that PD-L1 expression also in LSCC recurrences deserves closer examination.

We used clone SP142 instead of clone 22C3 as the former was in clinical use in our clinics when the study began. Clone 22C3 is preferred in PD-L1 diagnostics nowadays and has been developed as a selection marker for pembrolizumab. Accordingly, one TMA block served as a control and was stained with both antibodies. We found that PD-L1 expression was identical for both clones, SP142 and

22C3. The study utilized TMA blocks of T1 glottic LSCC. TMA blocks do not represent the whole tumor tissue and the small size of T1 glottic tumors causes challenges in TMA-block preparation.

Study III revealed that local recurrences and new primary tumors of the larynx were more common in patients with low stromal TIL density. TIL density was not associated with 5-year DSS in T1 glottic LSCC, even though all patients who died of LSCC had low stromal TIL density. In the previous studies as well, low TIL density was associated with poorer survival of LSCC.[140,141,149–154] Endpoints, recurrences and deaths, occur more rarely in T1 glottic LSCC, a fact which reduces the statistical power of the study. However, as a prognostic tool, TIL screening could still prove affordable and effortless.

The results of Study III indicate that significant immunological changes in the tumor microenvironment occur even in minor T1 glottic tumors. The tumor microenvironment comprises adjacent stromal cells, immune cells, stromal cells, blood vessels, and extracellular matrix.[110] Study III showed that the PD-1/PD-L1 pathway relating to T cell activation can be activated in T1 glottic LSCC, and TILs are often present. These results show that in T1 glottic LSCC the immune system is locally activated; possible systemic changes would be interesting to observe. Additionally, Study III showed, in T1 glottic cancer, a positive correlation between PD-L1 expression and TIL density. These findings are similar tho to those of studies investigating T1-T4 LSCC.[139,141]

Study III failed to find any association between TLR4, TLR5, and the prognosis of T1 glottic LSCC. Additionally, TLRs did not correlate with PD-L1, even though TLRs promote PD-L1 gene transcription via NF-κB. On the other hand, TLR4 and TLR5 have been studied more in oropharyngeal and oral SCC and high TLR5 expression may predict poor survival in HPV-positive oropharyngeal SCC.[169] Some TLRs are expressed in LSCC, but to our knowledge, any prognostic role for them in LSCC has not been apparent previously.[165,166]

Study III included only patients with T1 glottic LSCC. However, the earlier studies have comprised all stages and subsites of LSCC,

among which, T1 glottic LSCC differs significantly.[137–142] All five university hospitals in Finland participated in the study, making this study population rather representative. Additionally, Study III included patients undergoing both treatment modalities, corresponding to the real-life situation.

6.4 MMP-8 AND TIMP-1 IN RECURRENT RESPIRATORY PAPILLOMATOSIS AND LSCC

Study IV showed that elevated S-MMP-8 predicted malignant transformation of RRP, and showed high S-TIMP-1 to be associated with poor prognosis in LSCC. S-MMP-8 levels were higher both in RRP patients with malignant transformation and in LSCC patients compared to RRP patients without malignancy. Even in small T1 LSCC tumors, an elevation of S-MMP-8 levels occurred, and S-MMP-8 levels were higher than in RRP patients. High MMP-8 expression is associated with many inflammatory conditions, and our findings indicate that systemic inflammatory responses occur even in small laryngeal lesions.[171] In Study IV, S-MMP-8 levels correlated with tumor size, since S-MMP-8 levels were higher in advanced-stage LSCC than in early-stage LSCC. Inflammatory responses may be more intense in larger tumors.

In Study IV, malignant transformation occurred in both juvenile and adult-onset RRP. Patients with aggressive juvenile-onset RRP are also more susceptible to lung cancer. In adult-onset RRP, patients are typically older when malignant transformation occurs, and they more often present LSCC. Traditional risk factors, smoking and alcohol consumption, may play a more significant role in malignant transformation in such patients. In Study IV, the median time elapsed between serum sample collection and LSCC diagnosis in RRP patients with malignant transformation was 5 years (range 0.7-6.6 years. This indicates that S-MMP-8 level may predict malignant transformation many years before LSCC diagnosis.

Study IV showed that high S-TIMP-1 may be associated with unfavorable LSCC prognosis. To be more specific, elevated S-TIMP-1 was associated in multivariate analysis with poorer RFS and OS in LSCC. However, only multivariate analyses showed S-TIMP-1's association with prognosis, and S-TIMP-1 levels did not differ between the study groups since the results can be statistically underpowered. In the earlier studies, TIMP-1 predicted poor survival in HNSCC.[173–175] We are unaware of other studies examining S-TIMP-1, particularly in LSCC as a prognostic biomarker. In one study, TIMP-1 expression correlated with poorer OS in LSCC.[180] TIMP-1 stimulates tumor progression and spread by differing mechanisms. For instance, TIMP-1 can promote cell growth, inhibit apoptosis, and regulate angiogenesis.[192]

In Study IV, the number of RRP patients with malignant transformation was limited. All study patients were 40 years or older, meaning that the results can not be generalized to younger patients. However, LSCC and malignant transformation of RRP in this age group are, however, rare.

6.5 FUTURE PERSPECTIVES

Since the oncological outcome of T1 glottic LSCC with both TLM and RT is similar and good, the effects of treatment on quality of life should receive more intense consideration. Voice quality is an important aspect involved with glottic LSCC, and a study relating to this topic exists.[91] The quality of life in patients with total laryngectomy and posttreatment voice outcome in early glottic LSCC has been covered by several studies, but only a few studies concern the quality of life in T1 glottic LSCC patients.[193] One study including 123 T1a glottic LSCC patients showed that patient-reported quality of life did not differ between treatment groups (TLM vs. RT) in 1-year follow-up.[194] Another study included 91 T1-T2 glottic LSCC patients treated with TLM or RT. RT-treated patients showed higher emotional functioning and social contact on the European Organisation for Research and Treatment of Cancer questionnaires

than did patients treated with TLM.[195] For instance, T1 glottic LSCC patients' ability to work and their mental health need investigation.

The costs for RT are usually higher than for TLM, but the results are somewhat controversial.[92] For instance, the Dutch study showed the costs of TLM were higher than for RT when the costs of recurrences were included.[70] Health care systems and their financing differ between the countries, so the cost-effectiveness of treatment of T1 glottic LSCC is a good topic for invesigation in Finland. Research data concerning HNSCC follow-up protocols is limited, and therefore clinicians' experience is often essential in decision-making. The implementation of follow-up demands many health care resources, making it expensive. The cost-effectiveness of HNSCC follow-up should also be investigated in Finland.

Approximately 10%-50% of patients with laryngeal dysplasia develop LSCC during follow-up.[196] In Study I, of 303 patients, only 32 (11%) had been diagnosed with earlier dysplasia before their LSCC diagnosis. The mechanisms of malignant transformation of dysplasia are only in part yet recognized. For instance, the role of PD-L1, of infiltrating lymphocytes, and of S-MMP-8 could be relevant for study in laryngeal dysplasia.

The role of the PD-1 and PD-L1 pathway in RRP has been recently in focus. One study found that of 34 RRP tissue samples, 68% showed positive PD-L1 epithelial cell staining, and 76% contained PD-L1 positive infiltrating immune cells.[197] The PD-L1 antibody avelumab and PD-1 antibody pembrolizumab have been recently under examination in the treatment of RRP.[29] In phase II study, of nine patients with laryngeal RRP, six had a partial response to avelumab, and the number of surgical procedures was reduced during follow-up.[198] PD-L1's role in the malignant transformation of RRP could also be examined.

7 CONCLUSIONS

1. The prognosis of T1 glottic LSCC was favorable, and the treatment method (TLM vs. RT) did not affect it. The treatment decision should be considered individually, and the multidisciplinary tumor board meeting is important in recommending the best suitable treatment option for each patient.

2. The majority of T1 glottic LSCC recurrences were asymptomatic and were detected on a routine follow-up visit. Routine follow-up seemed beneficial in this patient group.

3. Low stromal TIL density was associated with the poorer prognosis of T1 glottic LSCC. The majority of T1 glottic LSCC tumors were positive for PD-L1, but PD-L1 played no prognostic role. In T1 glottic LSCC, PD-L1 expression correlated positively with TIL density.

4. Elevated S-MMP-8 predicted malignant transformation in RRP. High S-TIMP-1 may be associated with poor prognosis in LSCC.

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