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Tumor markers in T1-T2 laryngeal squamous cell carcinoma

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Tumor markers in T1-T2 laryngeal squamous cell carcinoma

Considerations on a clinical and biological level

J.E. Wachters

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Considerations on a clinical and biological level

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Introduction

Scope of this thesis

GENERAL INTRODUCTION

Epidemiology and etiology of head and neck cancer

Head and neck cancer encompasses a broad spectrum of malignancies, including mucosal and cutaneous squamous cell carcinomas, salivary gland tumors, melanomas, sarcomas and lymphomas. Annually, head and neck cancer is responsible for 550,000 new cases and 380,000 deaths worldwide.¹ Histologically, in the vast majority (approximately 90%) it concerns mucosal squamous cell carcinomas.

Head and neck squamous cell carcinoma (HNSCC) comprises a heterogeneous group of tumor entities. In the aerodigestive tract, anatomically, the paranasal sinuses, nasal cavity, oral cavity, pharynx and larynx can be distinguished. Depending on the HNSCC subregion, differences have been demonstrated in terms of etiology, clinicopathological behavior, epidemiology, diagnostic approach, treatment and prognosis. For example, nasopharyngeal squamous cell carcinoma is clinically characterized by, depending on the anatomical structures affected, epistaxis, nasal obstruction, auditory complaints and/or cranial nerve palsies. Risk factors include the consumption of salted fish and infection with Epstein-Barr virus and to a lesser degree human papilloma virus. As a risk factor, the role of tobacco and alcohol use is controversial. Generally, nasopharyngeal squamous cell carcinoma is treated primarily with (chemo)radiotherapy.^{2,3} Elsewhere in the HNSCC spectrum, hypopharyngeal carcinoma is clinically characterized by dysphagia, odynophagia, voice change and/or otalgia. Use of tobacco and especially alcohol are known risk factors. In general, hypopharyngeal squamous cell carcinoma is treated often with (chemo)radiotherapy with or without concurrent systemic therapy. However, a combination with surgery is frequently an option as well, depending on the stage of disease.^{4,5}

Because of this diversity of the anatomical subregions affected by squamous cell carcinoma, it is essential that HNSCC subdivisions are used to study and substantiate therapeutical strategies. Even an anatomical subregion such as the larynx, can be disaggregated into the supraglottis, glottis and subglottis for further substantiation (Figure 1 and 2). The supraglottic larynx includes the epiglottis, false vocal cords, ventricles, laryngeal aspect of aryepiglottic folds and arytenoids. The glottis includes the true vocal cords and the anterior and posterior commissure. The subglottic region begins 1 cm below the free edge of the true vocal cords and extends to the lower border of the cricoid cartilage (Figure 1). Of all HNSCC, about 21% is located in the larynx.⁶ In the Netherlands, the distribution of supraglottic, glottic and subglottic is 32.3%, 64.5% and 1.8% respectively. The remainder is comprised of LSCC of overlapping or unknown anatomical origin.⁷

Clinical characteristics and diagnosis of laryngeal cancer

Classically, laryngeal squamous cell carcinoma (LSCC), especially glottic LSCC, is characterized by a progressively hoarse or breathy voice. However, swallowing disorders may occur, predominantly in supraglottic LSCC. In more advanced stage LSCC, dyspnea, radiating earache and neck swelling may be present. At the moment of diagnosis supraglottic LSCC present more often with nodal metastasis.⁸⁻¹⁰

Figure 1. Anatomy of the larynx, mid sagittal overview of the aerodigestive tract and coronal laryngeal magnification. (figure partially adapted from Lewis et al. *Silverberg's Principles and Practice of Surgical Pathology and Cytopathology* 2015; 16:11-78-1203).ure.

Figure 2. Endoscopic view of the supraglottic, glottic and subglottic larynx, viewed from above.

For diagnosis, anamnesis, physical examination, including flexible laryngoscopy, biopsy and imaging (CT-, MRI-, PET- or ultrasound-imaging with or without aspiration cytology) is detrimental. Internationally, tumor size, node status and distant metastasis classification (TNM classification, maintained by the Union for International Cancer Control) is utilized, in order to study and to substantiate therapeutical strategies.

On the level of the supraglottis, a T1 tumor is limited to one subsite (epiglottis, false vocal cord, laryngeal ventricle etc.) with normal mobility of vocal cords while a T2 tumor extends to more than one subsite, without vocal cord fixation. A T3 tumor is limited to the larynx with vocal cord fixation and/or invasion of the inner cortex of the thyroid cartilage and/or infiltration of the pre-epiglottic space, paraglottic space or post-cricoid area.

On the level of the glottis, a T1a tumor is limited to one vocal cord and a T1b tumor extends to the contralateral vocal cord (via the anterior commissure), with normal vocal cord mobility. A T2 tumor extends to the supraglottis and/or subglottis and/or presents with impaired vocal cord mobility. A T3 tumor is limited to the larynx with vocal cord fixation and/or invasion of the inner cortex of the thyroid cartilage and/or infiltration of the paraglottic space.

On the level of the subglottis, a T1 tumor is limited to the subglottis and a T2 tumor extends to the vocal cords. A T3 tumor is limited to the larynx with vocal cord fixation and/or invasion of the paraglottic space and/or inner cortex of the thyroid cartilage.

For both supraglottic, glottic and subglottic LSCC, a T4a tumor, also called “moderately advanced local disease”, invades through the thyroid cartilage and/or invades tissues beyond the larynx (such as trachea, soft tissues of the neck including deep extrinsic muscle of the tongue, strap muscles, thyroid gland or esophagus). “Very advanced local disease”, a T4b tumor, invades the prevertebral space, encases the carotid artery and/or invades mediastinal structures.

For all LSCC, the situation considering regional lymph node metastasis can be described by means of the N status. When no regional lymph node metastasis can be proved, the status is N0. N1 means metastasis to a single ipsilateral lymph node, 3 cm or less in greatest diameter. N2a and N2b describes a situation with metastasis to a single or to multiple ipsilateral lymph node(s) respectively, more than 3 cm but not more than 6 cm in greatest dimension. N2c describes a situation with metastasis to bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension and N3 describes metastasis to a lymph node, more than 6 cm in greatest dimension. M-status describes the presence of distant metastasis. M1 and M0 describe a situation with and without the presence of distant metastasis respectively.

Treatment

The aim of LSCC treatment is to achieve local and/or locoregional control of the disease with preservation of laryngeal function (speech, swallowing, pressure build-up for coughing and core-stability). According to the NCCN Guidelines Version 1.2019, for both glottic and supraglottic T1-T2 N0 and selective T3 N0 cases, 2 options are proposed: radiotherapy or surgery (endoscopic surgery, open surgery, such as partial laryngectomy, with or without neck dissection).¹¹ Open surgery is very rarely indicated and in The Netherlands only occasionally performed in early stage LSCC. In short, it involves an open neck wound with partial or total surgical laryngeal resection with or without insertion of a voice prosthesis device. Open surgery is accompanied by a hospital admittance of at least several days to weeks and by complications such as wound infection and fistula formation.

According to the Dutch guideline Laryngeal Carcinoma, endoscopic surgery (CO₂-laser excision) is the preferred treatment modality for T1a glottic LSCC.¹² In case of endoscopically inoperable relapse, or in case of all other T1-(small)T3 N0 or N1 LSCC, radiotherapy ultimately is a main

treatment modality. In case of T1-T3 N2-N3 LSCC, concurrent systemic therapy (such as cisplatin or carboplatin/ 5-FU) combined with radiotherapy is applicable. Cetuximab treatment can be applied when chemotherapy is contraindicated.¹¹ This treatment is recommended for non-resectable T4 and resectable T4 LSCC with expected preservation of laryngeal function after treatment as well. However, for resectable T4 LSCC without expected preservation of laryngeal function after treatment, total laryngectomy with neck dissection and postoperative (chemo)radiotherapy is recommended. In case of metastatic disease at initial presentation (M1) comparable options are recommended with additionally, when possible, locoregional treatment based on primary algorithms.¹¹

Radiotherapy

Radiotherapy is, in both late- and early-stage LSCC, a prominent treatment modality. Radiotherapy utilizes ionizing radiation, which is applied on tumor tissue, damaging nuclear DNA directly, but indirectly by initiating the formation of free radicals as well. Oxygen molecules react with these free radicals and stabilize the desired double-strand breaks in nuclear DNA, leading to loss of reproductive ability of the tumor cells and eventually to cell death. Increasing the dose of ionizing radiation increases the tumor tissue demise. However, toxic side effect such as oral mucositis, dysphagia, radiation skin burns and xerostomia can occur due to damage to surrounding tissues. In order to maximize tumor tissue exposure and to minimize exposure of surrounding tissue, radiation beams are applied using varying shapes and application angles. Moreover, radiotherapy is given in a fractionated scheme in order to allow surrounding tissues to recover.¹³ In early stage LSCC, traditionally 5 days a week fractions of 1.8-2.0Gy were administered during a treatment course of 7 weeks, resulting in a total dose of 66-70Gy. However, during the period this thesis encompasses, accelerated radiotherapy was introduced in order to prevent potential tumor cell repopulation processes during the treatment course. With 6 days a week, daily fractions of 2.0Gy during a 6 week treatment course, toxic side effects increase, but local control and survival rates as well.¹³ Accelerated radiotherapy was implemented in our institute for T2b-T4 glottic and T2-T4 supraglottic LSCC patients from the year 2000 onward.

Reported local control rates in early stage LSCC after radiotherapy vary widely (61-100%), as do 5-year disease specific survival rates (76-100%).¹⁴

In advanced stage LSCC, clinical outcome/survival is worse than in early stage LSCC. Protocols of (chemo)radiotherapy in order to facilitate laryngeal preservation have been developed in the 1990's.^{15, 16} Ever since, the survival in advanced stage LSCC did not improve, possibly because of these protocols.¹⁷

Radiotherapy failure

Locoregional recurrent or residual tumor growth after radiotherapy in early and advanced stage LSCC, whether or not preceded by surgery, most frequently results in challenging and extensive salvage surgery. This most often comprises partial or total laryngectomy with or without neck dissection and muscle flap reconstruction. Radiotherapy has been described in the literature to impair wound healing and therewith has been correlated with a highly morbid postoperative course.^{18, 19} A major wound complication rate of 60% and a pharyngocutaneous fistula rate of 30%

have been described.¹⁸ Most often additional surgical procedures and hence prolonged hospital admissions are required. Moreover, survival can be poor, with reported 2- to 5-year overall survival of 19 to 36%.²⁰

The TNM staging system is not of sufficient predictive value towards local and/or locoregional control in LSCC treated with radiotherapy. Hence, to be able to prevent recurrent disease or unnecessary radiotherapy induced adverse effects, in other words, for appropriate treatment decision-making in LSCC, there is a need for new tumor markers with predictive value.²¹⁻²³ According to the NCI Dictionary of Cancer Terms, a tumor marker can be defined as a biological molecule found in blood, other body fluids or tissues that is a sign of a normal or abnormal process or of a condition or disease; and may be used to see how well the body responds to a treatment for a disease or condition.²⁴ Using tumor markers, subselections of patients might be identified, that benefit from more aggressive treatment modalities. Since several decades numerous research groups have been trying to identify predictive and prognostic markers for radio response in LSCC based upon physical, molecular and genetic hypotheses.^{25, 26}

In HNSCC, using a variety of techniques like immunohistochemistry, CT, MRI and PET imaging, several markers have been proposed and related to potentially outcome deteriorating processes such as tumor hypoxia, chromosome 11q13 amplification and tumor associated signaling pathway alterations in HNSCC,^{21, 27-30} as will be discussed in the following paragraphs.

Tumor hypoxia

Tumor hypoxia is a common phenomenon in solid tumors and can be caused by a variety of mechanisms. Chronic hypoxia can be caused by diffusion limitations due to overgrowth of tumor cells and concomitant insufficient vascularization, or hypoxemia due to heavy use of tobacco. Acute hypoxia can be caused by transient blood vessel disruptions due to a chaotic network of vessels in tumors.²⁷ Tumor hypoxia has been linked to resistance to chemo- and radiotherapy. Due to hypoxic circumstances, therapy-induced damage to nuclear DNA is inhibited.^{31, 32} Furthermore, especially chronic tumor hypoxia induces tumor cells to be more invasive and to metastasize more easily, resulting in a poorer prognosis by its reported influence on pathways regulating a variety of essential processes such as angiogenesis, growth-factor signaling, cell migration, pH regulation and apoptosis.^{31, 33}

Measurement of tumor oxygenation status in solid tumors was demonstrated in the early nineties, using polarographic tumor micro-electrodes (Eppendorf device) in a variety of solid tumors and is still considered the gold standard for measurement of tumor hypoxia.³⁴ In HNSCC associations between low tumor oxygenation status were found with overall survival and local control.³⁵ However, the invasive nature of the technique, the rather numerous necessary measurements and the lack of resolution within the tumor, kept the technique from implementation in clinical routine.

Tumor hypoxia can be assessed by measuring the presence of endogenous hypoxia markers, specific protein expression profiles known to be upregulated by hypoxia, using immunohistochemistry.^{27, 34} A central role in the cellular response to hypoxia has been laid out for a group of heterodimeric transcription factors called Hypoxia Inducible Factor (HIF) that is upregulated in response to hypoxia.^{27, 34} One of these transcription factors (HIF-1a) plays a key role.

In normoxic conditions HIF-1 α is hydroxylated and thereby degraded. Under hypoxic conditions this degradation is inhibited, enabling dimerization of HIF1 α with HIF-1 β , consequent binding to hypoxia response elements and ultimately regulation of expression of Carbonic Anhydrase-IX (CA-IX), Glucose Transporter-1 (GLUT-1), Vascular Endothelial Growth Factor (VEGF) and Erythropoietin (EPO), all known to promote adaptations to hypoxic conditions (Figure 3).^{27, 34, 35} Osteopontin (OPN) is thought to be a HIF-independent upregulated potential marker of hypoxia, known to play a role in bone remodeling, immune response and inflammation, but ultimately in cell proliferation, invasion and angiogenesis as well.³⁶

Although there is a lack of association between endogenous markers of hypoxia and polarographic needle electrode measurements in general, there are many reports on the prognostic and/or predictive value of endogenous hypoxia markers in HNSCC cohorts.^{27, 34, 35} However, diversity in immunohistochemical technique, anatomical sublocalization, TNM stage and treatment strategy lead to conflicting and divergent results, that keep these markers from being incorporated in daily strategies.^{27, 34, 35} Evaluation of endogenous hypoxia markers in a large homogeneous cohort considering HNSCC sublocalization and TNM staging, uniformly treated and immunohistochemically analyzed, seems necessary to overcome these problems. For this reason, our research group

previously investigated the predictive value of HIF-1a, CA-IX and GLUT-1 in a homogeneous group of 91 T1-T2 glottic LSCC, all treated with radiotherapy only. Both HIF-1a and CA-IX were of predictive value toward local control.³⁷

Tumor hypoxia can be evaluated by the use of (intravenously injectable) exogenous markers, such as pimonidazole and 2-nitroimidazol-pentafluoropropyl acetamide (EF5), 18F-fluoromisonidazole (F-FMISO) and 18F-fluoroazomycin arabinoside (F-FAZA). Under hypoxic conditions intracellularly ($pO_2 < 5-10$ mmHg), these compounds are reversibly reduced to highly reactive oxygen radicals that easily bind to intracellular macromolecules. Reduced and bound pimonidazole and EF5 are then detectable after tumor biopsy and immunohistochemical treatment using specific immunohistochemistry.³⁸ In a similar way, F-FAZA and F-FMISO are detectable, not by immunohistochemistry, but by Positron-Emission Tomography (PET-scan).²⁸ Compared to biopsy driven immunohistochemistry, a potential advantage of PET-scanning is the possible detection of intratumoral heterogenic areas of hypoxia. Only a weak association was found between F-FMISO PET-scanning and polarographic needle electrode measurements.³⁹ More convincingly, increased F-FMISO uptake in HNSCC has been linked to a negative prognosis.³¹

Drawbacks of F-FMISO are its relative lipophilicity which counteracts sufficient uptake, and its relatively short biological half-life-time (50 minutes), which impairs contrast within PET-scan images (target-to-background-ratio). F-FAZA on the other hand is less lipophilic and cellular uptake might take place more easily. Moreover, its biological half-life-time is 4 days, potentially enhancing target-to-background ratios.^{31, 40} Few studies concerning F-FAZA uptake in HNSCC have been performed. Only Mortensen et al. investigated relations with clinical outcome and demonstrated F-FAZA uptake to be related with poorer prognosis in 40 HNSCC patients treated with (chemo)radiotherapy.⁴¹ The benefit of a F-FAZA and F-FMISO PET-scan is the possibility to evaluate the degree of hypoxia in a total tumor mass, instead of immunohistochemical evaluation of only small fields of tumor in biopsies with potentially questionable hypoxic representativity. However, studies evaluating relations of hypoxia measured by endogenous markers and radiopharmaceutical driven imaging techniques within these hypoxic tumor fields in the same tumor tissues have not been performed.²⁸

Amplification of chromosome 11q13 and increased expression of relevant genes

Amplification of chromosomal DNA has been described in a variety of human malignancies and is generally considered a mechanism enabling selective overexpression of genes and implicating a selective advantage for these particular cells. The 11q13 chromosome region is amplified most often in HNSCC (36%)^{42, 43} and has been associated with aggressive tumor growth and impaired clinical outcome.⁴⁴ The 11q13 amplicon contains 13-14 genes including CCND1, CTTN and FADD, encoding for the proteins Cyclin D1, Cortactin and Fas-associated Death Domain (FADD), respectively, that are commonly amplified and overexpressed in HNSCC.⁴⁵

The FADD-protein contains a "death domain", enabling binding to "death effector domain" containing proteins, such as Procaspase-8 and -10. These protein complexes have a key role in mediating apoptosis. However, FADD can be phosphorylated at a specific serine residue (Ser-194), which is associated with nuclear localization, predominantly in the G2/M phase of the cell cycle, suggesting that phosphorylated FADD (pFADD) is involved in cell cycle regulation and

cell proliferation.⁴⁶ Pattje et al. demonstrated that expression of pFADD was a strong prognostic factor for local control in HNSCC patients treated with surgery and postoperative radiotherapy. In concordance with these findings, in vitro experiments demonstrated an increased radiosensitivity in HEK293 cell lines overexpressing pFADD, implicating a key role in cell cycle control and ultimately in clinical outcome.⁴⁷ Also involved in cell cycle regulation is Cyclin D1, which phosphorylates, thereby inactivates the Retinoblastoma protein (pRb) and promotes G1-S transition in the cell cycle and thus cell proliferation. In HNSCC a meta-analysis suggested that cyclin D1 overexpression could represent an important prognostic factor.⁴⁸

Cortactin is an actin-binding protein, which regulates, amongst others cell-cell adhesion, actin-network assembly and membrane degradation. This results in effecting motility and invasive potential via regulating invadopodia and lamellipodia in the cell membrane.^{30, 49} Expression of cortactin has been associated with the metastatic potential in HNSCC patients.^{30, 50-52}

In LSCC, the increased expression of (p)FADD, cyclin D1 and cortactin demonstrates significant associations with clinical outcome, but with negative results as well.^{30, 53-59} Probably these inconsistencies are due to inhomogeneous study populations considering TNM-staging, treatment and tumor sublocalization.^{50, 53, 54} Therefore, our research group previously investigated the predictive value of FADD, pFADD, cyclin D1 and cortactin in the same homogeneous T1-T2 LSCC population all originating from the glottis and treated with radiotherapy only. In this cohort, only expression of pFADD was significantly associated with local control.⁶⁰

The EGFR-PI3K-AKT pathway

A whole-exome sequencing study demonstrated that in HNSCC the EGFR-PI3K-AKT pathway was the most frequently mutated oncogenic pathway (>30%).⁶¹ The Epidermal Growth Factor Receptor (EGFR) is a cell-surface receptor, potentially activated by multiple ligands, which in turn leads to activation of Phosphoinositide 3-kinase (PI3K). Activated PI3K promotes the formation of Phosphatidylinositol-3,4,5-triphosphate (PIP3). On the other hand, formation of PIP3 is antagonized by Phosphate and Tensin homolog deleted on chromosome 10 (PTEN). PIP3 translocates Protein kinase B, also known as AKT, to the plasma membrane, in order to realize phosphorylation and hence activation of AKT (referred to as pAKT). This phosphorylation of AKT can take place at 3 regulatory sites (Thr308, Ser 473, Ser129) by a variety of kinases. Phosphorylated AKT results in cell survival and cell proliferation (Figure 4).⁶² Since activation of EGFR indirectly promotes the phosphorylation of AKT and hence tumor progression, the role of EGFR in cancer has been under investigation for years. In HNSCC, EGFR is commonly overexpressed (>80%) and has been linked to radioresistance.^{22, 29, 62, 63} However, the prognostic/predictive value of EGFR overexpression towards clinical outcome parameters such as locoregional recurrence or overall survival in HNSCC is still under debate.^{25, 64, 65} PTEN is thought to be a tumor-suppressor gene by antagonizing the formation of pAKT, since loss of PTEN, leads to permanent activation of the EGFR-PI3K-AKT pathway.^{61, 65, 66} PTEN has, however, also been linked to DNA double strand break repair and hence genomic stability by regulating RAD51.^{61, 65, 66} Since the effect of radiotherapy is based on a high proliferation rate and inhibited DNA repair compared to surrounding healthy tissues, theoretically PTEN overexpression could lead to radioresistance, as confirmed by the findings of Pattje and coworkers in HNSCC treated with surgery

and radiotherapy.⁶⁵ However, Snietura and coworkers demonstrated an association between loss of PTEN expression and a worse locoregional control in HNSCC treated (postoperatively) with radiotherapy.⁶⁷ Therefore, the predictive/prognostic value in HNSCC of PTEN expression is still unclear. To elaborate on the predictive and prognostic value of the expression of EGFR and PTEN in HNSCC, evaluation of the expression of these markers needs to be conducted on independent homogeneous populations regarding tumor sublocalization, TNM-stage and therapy modality.

Figure 4. The EGFR-PI3K-AKT pathway and its (patho)physiological effects. (figure partially adapted from Horn D et al. *Expert Opin Ther Targets* 2015;19(6):795-805).

SCOPE OF THIS THESIS

For appropriate treatment decision-making in HNSCC, there is a need for new tumor markers to distinguish patients with poor and good clinical outcome. In T1-T2 LSCC treated with only radiotherapy, 0-39% develop local recurrent disease with often devastating consequences. These patients might benefit from a more aggressive treatment. Many markers have been investigated as potential predictive or prognostic markers, but these studies were often performed on inhomogeneous cohorts regarding HNSCC sublocalization, TNM-stage and treatment modality. This might be an explanation for the divergent results found in other studies. On a laryngeal level, glottic and supraglottic LSCC demonstrate differences in clinical presentation and clinical behavior, and might therefore represent different entities. As such, tumor markers might be of a

different predictive/prognostic value within these subregions. In this thesis, a well-defined cohort of supraglottic LSCC patients was selected. All patients were diagnosed with and/or treated for a histologically confirmed T1 or T2 supraglottic LSCC in the University Medical Center Groningen between 1990 and 2011. All patients were treated with primarily radiotherapy only, with curative intent. Previously, our research group studied the predictive/prognostic value of several tumor markers in a homogeneous cohort of T1-T2 glottic LSCC with otherwise identical inclusion criteria. In this thesis, tumor marker characteristics in supraglottic LSCC are compared with our earlier results on glottic LSCC.

Chapters 2 and 4 focus on the predictive/prognostic value of endogenous markers associated to tumor hypoxia and 11q13-amplification in T1-T2 supraglottic LSCC treated primarily with radiotherapy. Results are compared to our previously reported findings in a homogeneous cohort of T1-T2 glottic LSCC treated with radiotherapy only.^{37,60}

Chapter 3 encompasses a study investigating the accuracy of F-FAZA-PET/CT scanning and comparing this technique with endogenous and exogenous hypoxia markers, in advanced stage and recurrent LSCC patients. In these same patients hypoxia markers (pimonidazole, HIF-1a, CA-IX and GLUT-1) are evaluated by immunohistochemistry and in-situ compared with results of F-FAZA-PET/CT scanning with the aim to determine spatial hypoxic regions.

In chapter 5, the association between local control upon radiotherapy and EGFR, a widely studied marker in various types of cancer and a potential target for adjuvant therapy, is evaluated in both glottic and supraglottic early stage LSCC, treated with radiotherapy only. In parallel, PTEN, known as a tumor marker involved in both tumor suppression, as well as in DNA repair, is evaluated in our supraglottic cohort.

Finally, given the established epidemiological and clinical differences between glottic and supraglottic LSCC and the potential differences in predictive/prognostic value of markers, in chapter 6, the expression patterns of 11 tumor markers that are involved in, amongst others, tumor hypoxia, chromosome 11q13 amplification, EGFR-PI3K-AKT pathway are analyzed. Differences between T1-T2 glottic and supraglottic LSCC are evaluated.

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Prognostic significance of HIF-1a, CA-IX and OPN in T1-T2 laryngeal carcinoma treated with radiotherapy

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ABSTRACT

Objectives: to examine the prognostic value of hypoxia inducible factor HIF-1a, CA-IX and OPN on clinical outcome in patients with T1-T2 supraglottic LSCC treated with primarily radiotherapy (RT).

Study design: retrospective cohort study (level of evidence 2b).

Methods: tumor tissue sections of 60 patients with T1-T2 supraglottic LSCC treated with primarily radiotherapy were assessed immunohistochemically for expression of HIF-1a, CA-IX and OPN. The relationship of protein expression and classical clinical parameters with clinical outcome was studied, using Cox regression and Kaplan-Meier survival analyses.

Results: neither HIF-1a nor CA-IX was of prognostic significance towards local control or overall survival in T1-T2 supraglottic LSCC. Cox regression survival analysis showed no relation between HIF-1a or CA-IX expression and local control (HR [hazard ratio] 1.07, CI [95% confidence interval] 0.29-3.87; HR 0.34, CI 0.04-2.58). Furthermore, OPN expression was not associated with local control (HR 1.37, CI 0.45-4.17) and overall survival (HR 0.99, CI 0.44-2.21). Our earlier findings in T1-T2 glottic LSCC (Schrijvers et al., 2008) could not be confirmed.

Conclusion: The absence of prognostic significance for HIF-1a and CA-IX towards local control in supraglottic LSCC, unlike glottic LSCC, suggests that supraglottic LSCC might represent another biological entity.

INTRODUCTION

Head-and-neck cancer represents the sixth most common cancer worldwide with approximately 650,000 new cases annually¹. About 25% of these tumors is located in the larynx and the vast majority of laryngeal tumors is of squamous cell origin (~90%)^{2,3}.

Treating laryngeal squamous cell carcinoma (LSCC) by surgery through a partial or total laryngectomy results in high overall survival accompanied by a major decline in laryngeal function and hence in quality of life. Radiotherapy (RT) has the advantage that it preserves laryngeal function⁴ with a more or less equal survival in T1-T2 LSCC^{5,6}. However, reported local control rates after RT vary between 63%-91% and 41%-100% for glottic and supraglottic T1-T2 LSCC respectively^{4,7-9}. Recurrent disease after RT results in salvage surgery through total laryngectomy accompanied by high rates of morbidity and mortality. In order to prevent this complicated course of disease it is essential to be able to predict the efficacy of RT. At present, in order to predict RT treatment response, clinicians rely upon TNM staging. Unfortunately, RT failures in early stage LSCC are not accounted for by the current system.

A well-known adverse prognostic factor in head-and-neck squamous cell carcinoma (HNSCC) is tumor hypoxia which is a common phenomenon in solid tumors¹⁰. Several methods have been used for measuring tumor oxygenation status, including polarographic tumor microelectrodes, exogenous markers (e.g. pimonidazole), nuclear imaging techniques (e.g. F-FAZA) and endogenous hypoxia markers (e.g. hypoxia inducible factor [HIF]-1a, carbonic anhydrase [CA]-IX, osteopontin [OPN]).

We previously demonstrated in a group of 91 T1-T2 glottic LSCC primarily treated with RT that expression of HIF-1a and CA-IX were of prognostic value towards local control¹¹. Combining HIF-1a and CA-IX expression, an even stronger prognostic profile was found. Although in the literature HIF-1a and CA-IX expression have been associated predominantly with impaired clinical outcome in HNSCC, a number of studies found no or an adverse relationship between clinical outcome and HIF-1a and CA-IX expression¹²⁻¹⁷. These contradicting results between various independent studies might partly be explained by a lack of uniformity in study populations, especially considering tumor localization and its corresponding differences in clinical behaviour¹⁸. In this study, we therefore investigated the prognostic value of HIF-1a and CA-IX in a homogeneous group of T1-T2 supraglottic LSCC, treated with primarily RT, enabling a comparison with our earlier findings in glottic LSCC. In addition, the prognostic value of classical prognostic markers of clinical outcome such as gender, age, primary complaint, T-status, N-status and pre-treatment hemoglobin level will be investigated.

In the literature HIF-1a is demonstrated to be upregulated by other factors than hypoxia¹⁹. Therefore, we hypothesize that the use of an HIF-1a independent endogenous hypoxia marker could be of additional value. OPN is thought to be upregulated during hypoxia independently of HIF-1a²⁰. OPN expression has been linked to tumor hypoxia in HNSCC treated with varying treatment modalities and has been associated with unfavourable overall and 5-year survival²¹⁻²³, but has not been tested as a prognostic marker for local control after RT in LSCC. Therefore, we also investigated the additional prognostic significance of OPN expression in T1-T2 supraglottic LSCC.

MATERIAL AND METHODS

Patients

Retrospectively a database was constructed covering 1286 patients diagnosed with and/or treated for laryngeal malignancies in the University Medical Center Groningen between 1990 and 2008. Medical records of all patients were reviewed, collecting clinical, histopathological and follow-up data.

The inclusion criteria for this study were histologically proven LSCC, restricted to the supraglottic region only, stage T1 or T2, curatively treated with RT with no other prior treatment, of which pre-treatment, formalin-fixed and paraffin-embedded tumor material was available.

Our database contained 247 patients with T1-T2 LSCC. Ten patients were excluded because of prior regional RT, chemotherapy or concurrent second primary malignancies. All pre-treatment biopsy slides were revised and tumor percentages were estimated by an experienced pathologist. Of 237 remaining biopsy specimens, 145 were found to contain sufficient tumor tissue for immunohistochemical staining, consisting of 60 supraglottic LSCC patients.

The pre-treatment parameters are presented in table 1. Patients were clinically followed every 3 months for 2 years and every 6 months up till 5 years after completing RT.

At the date of analysis, median follow-up time was 43.5 months (range 5-169). Thirteen (21.7%) of 60 patients developed a local recurrence, of which 4 patients (6.7%) received palliative or no further treatment and 9 patients (15.0%) received a total laryngectomy with or without a neck dissection. The median time to local recurrence was 13.0 months (range 5-18). Twenty-six out of 60 patients (43.3%) deceased, of which 8 (30.8%) as a consequence of their LSCC. The median time to death was 38.0 months (range 6-154) (table 2).

Treatment

All patients included in this study were primarily treated with RT only. RT was administered using megavoltage equipment (6 MV photons) and thermoplastic masks. Before 2000, field arrangements were set by direct simulation. After 2000, contrast-enhanced CT-scans were used for planning.

In T1-T2aN0 cases, clinical target volume generally consisted of the gross tumor volume with 1 cm margins. In case of direct simulation, the field borders were set at the lower edge of the hyoid bone and the lower border of the cricoid cartilage. These patients were treated with two opposing lateral fields with a median fraction dose of 2.0Gy (5 times/week) up to 66-70Gy in 7 weeks.

In the other T2 cases or in case of T1-T2/N+, the initial clinical target volume consisted of the primary tumor, the pathological lymph nodes plus 1 cm margins, and bilateral elective nodal areas. The boost volume consisted of the tumor and positive lymph nodes with 0.5 cm margins. For the planning target volume (PTV) 0.5 cm margins were applied. In general, patients were treated using 2 opposing lateral fields for the upper neck nodes and the primary site. PTV1 was treated with a total dose of 46Gy, followed by a boost dose of 24Gy to the primary tumor and pathological lymph nodes.

Most patients were treated with conventional fractionation, however, from 2000, patients were generally treated with 6 fractions/week with a second fraction on Friday afternoon with a minimum interval of 6 hours, up to 70Gy in 6 weeks.

Table 1. Pretreatment clinical and immunohistochemical characteristics (n=145).

Parameter	Subgroup	Supraglottis n=60 n (%)
Gender	-Male -Female	43 (71.7) 17 (28.3)
Age (years)	-Median (Range) -≤ 64 ->64	62.0 (33-96) 39/60 (65.0) 21/60 (35.0)
Primary symptom	-Hoarseness -Other -Unknown	32/60 (53.3) 28/60 (46.6) 0/60 (0.0)
T-status	-1 -2	16/60 (26.7) 44/60 (73.3)
N-status	-0 ->0 -1 -2 -3 -x	45/59 (76.3) 14/59 (23.7) 9/59 (15.3) 4/59 (6.8) 1/59 (1.7) 1/60
Hemoglobin (mmol/l)	-Median (Range) -Low -High -Unknown	9.0 (6.7-15.4) 14/58 (13.9) 44/58 (75.9) 2/60
HIF-1a expression	-Median (Range) -Low -High	6.0 (0-50) 13/60 (21.7) 47/60 (78.3)
CA-IX expression	-Median (Range) -Low -High	1.0 (0-40) 49/60 (81.7) 11/60 (18.3)
OPN expression	-Median (Range) -Low -High	0.0 (0-80) 40/60 (66.7) 20/60 (33.3)

Immunohistochemistry

Four µm serial sections were cut and hematoxylin and eosin staining was performed to verify histology. The slides were deparaffinized using xylene, rehydrated through a series of decreasing ethanol dilutions and phosphate buffered saline (PBS). The details of the immunohistochemical stainings are presented in table 3. In short, after antigen retrieval was performed endogenous peroxidases were blocked for 30min. at room temperature with 0.3% H₂O₂. The sections were incubated with the primary antibodies followed by the secondary and tertiary antibodies. Subsequently, the peroxidase reaction was performed by applying 3,3'-diaminobenzide tetrachloride for 10min. Finally, after washing the slides with PBS, they were counterstained with hematoxylin for 2min., dehydrated and mounted.

Evaluation of immunohistochemical staining

All slides were scored by 2 observers separately, both unaware of clinical follow-up data. All differences were analyzed and resolved with conference microscope sessions. The percentage of tumor cells stained was evaluated. As previously reported, for HIF-1a, CA-IX and OPN only

Table 2. Follow-up data.

Follow-up parameter	Subspecification	Supraglottis (n=60) n (%)
Events in follow-up	-Patients with any event -Local recurrence -Reg. recurrence -2nd primary head/neck region -Death Death of disease Death not of disease Unknown	30/60 (50.0) 13/60 (21.7) 1/60 (1.7) 1/60 (1.7) 26/60 (43.3) 8/26 (30.8) 16/26 (61.5) 2/26 (7.7)
Time to local recurrence (months)	-Median (Range)	13.0 (5-18)
Time to death (months)	-Median (Range)	38.0 (6-154)

Table 3. Details on immunohistochemical stainings.

Antigen	OPN	HIF-1α	CA-IX
Primary antibody	Anti-OPN 1:50, 60 min, RT ^e	Anti-HIF-1α 1:100, ON ^f , 4 °C	Anti-CA-IX 1:500, 60 min, RT
Species	Rabbit	Mouse	Mouse
Source	Thermo Fisher Scientific ^a	BD Biosciences ^c	Dr. J. Pastorek ^d
Antigen Retrieval	Citrate pH 6.0 100°C, 15 min	Tris/EDTA ^g pH 9.0 100°C, 15 min	-
Secondary antibody	GAR ^{PO} ^b 1:300, 30 min, RT	RAM ^{BIO} ^b 1:300, 30 min, RT	RAM ^{BIO} ^b 1:300, 30 min, RT
Tertiary antibody	RAG ^{PO} ^b 1:100, 30 min, RT	ABC ^{HRP} ^b 1:100, 30 min, RT	ABC ^{HRP} ^b 1:100, 30 min, RT
Localization of staining	Cytoplasmic	Nuclear	Membranous

^a Thermo Fisher Scientific, Fremont, USA, ^b Dako, Glostrup, Denmark, ^c BD Biosciences, New Jersey, USA,

^d Jaromir Pastorek, University of Bratislava, Slovakia, ^e RT room temperature, ^f ON overnight, ^g Tris/EDTA tris/ethylenediaminetetraacetic acid buffer.

nuclear^{13,24,25}, membranous^{11,24,26-28} and cytoplasmatic²¹⁻²⁴ staining respectively were evaluated. For evaluation, we used cut-off values of 0.5%, 12.5% and 0.5% stained tumor cells respectively.

Statistical analysis

All statistical analyses were performed using the SPSS 16.0 software (Chicago, USA). Correlations between expression percentages were calculated using the Spearman's rank correlation coefficient. Using logistic regression analysis, the associations between expression percentages and clinical

characteristics were analyzed.

Time calculations were performed using the date of diagnosis as starting point and the day of local recurrence, death or last follow-up visit as endpoint. Local recurrence was defined as reappearing tumor growth at the primary tumor site after treatment.

Survival curves were plotted according to the Kaplan-Meier method and the log-rank test was used to determine whether curves crossed. Univariate/multivariate Cox proportional hazard models were used to assess which tumor variables were independently correlated with death and local recurrence. In all statistical analyses, a p -value ≥ 0.05 was considered to be statistically significant.

RESULTS

Immunohistochemistry and scoring evaluation

The HIF-1a, CA-IX and OPN, stainings were rather homogeneous in intensity and almost exclusively

Figure 1. Positive staining for HIF-1a(A), CA-IX(B) and OPN(C). Original magnification, 200x.

nuclear, membranous and cytoplasmic respectively, as demonstrated in fig 1a-c. The mean percentage positivity was 9.2%, 5.8% and 4.7% respectively (table 1).

Associations among immunohistochemical and clinical variables

The Spearman's correlation test demonstrated for HIF-1a and CA-IX expression percentages significant correlations ($r=0.46$ $p<0.001$). OPN was not associated with HIF-1a and neither with CA-IX ($r=0.21$ $p=0.11$; $r=-0.06$ $p=0.65$ respectively).

Using logistic regression, no significant associations were found between expression percentages and clinical characteristics including gender, age, primary symptom, T-stage, N-stage and hemoglobin level (data not shown).

Associations between immunohistochemical/clinical variables and treatment outcome

Univariate Cox regression analysis showed that only N-status was significantly associated with local control (HR 3.22, CI 1.08-9.62). However, age and T status were associated with overall survival

(HR 3.41, CI 1.50-7.71; HR 2.92, CI 1.06-8.09 respectively)(table 4-5). In multivariate Cox regression analysis both parameters appeared to be of independent prognostic value (HR 3.29, CI 1.44-7.51; HR 2.84, CI 1.06-8.09 respectively).

Univariate Cox regression analysis revealed that both local control and overall survival could not be predicted by elevated HIF-1a expression (HR 1.07, CI 0.29-3.87 and HR 1.23, CI 0.42-3.64 respectively) and neither by elevated CA-IX expression (HR 0.34, CI 0.04-2.58 and HR 0.83, CI 0.31-2.21 respectively)(table 4 and 5).

After combining HIF-1a and CA-IX into a hypoxic profile (defined as high HIF-1a and/or high CA-IX expression as we reported previously¹¹), no associations were found with local control and overall survival (HR 1.07, CI 0.29-3.87 and HR 1.23, CI 0.42-3.64 respectively)(table 4 and 5, figure 2a).

OPN expression was also not of predictive value towards local control and overall survival (HR 1.37, CI 0.45-4.17 and HR 0.99, CI 0.44-2.21 respectively). Insertion of OPN expression into the hypoxic profile (defined as low HIF-1a, low CA-IX and low OPN expression) did not result in an improvement of the predictive value of this combined marker approach (table 4 and 5, fig 2b).

DISCUSSION

In this study we investigated the prognostic value of classical pre-treatment parameters and endogenous hypoxia markers HIF-1a, CA-IX and OPN in T1-T2 supraglottic LSCC. Additionally, we compared this with our previous findings in T1-T2 glottic LSCC¹¹, both treated with RT only.

Tumor hypoxia has been shown to be of importance in promoting genetic instability, tumor cell invasiveness, metastasis and treatment failure in HNSCC^{10,29}. Several methods have been described to measure tumor oxygenation status. Measuring tumor hypoxia directly, using polarographic microelectrodes, exogenous markers (e.g. pimonidazole) or nuclear imaging techniques (e.g. F-FAZA), has limitations due to invasiveness or necessity for intravenous pre-injection. However, microelectrode measurements are considered to be the gold standard³⁰. In HNSCC treated with a variety of treatment modalities including RT, hypoxia as measured with microelectrodes, has been associated with diminished locoregional control^{31,32} and overall survival^{33,34}. More indirectly, hypoxia can be assessed in pre-treatment tumor biopsies by immunohistochemically measuring the presence of endogenous markers, specific protein expression profiles known to be upregulated by hypoxia²⁰. Endogenously measuring tumor hypoxia has been mainly focussed on the presence of HIF-1a and its downstream regulated genes (e.g. CA-IX, GLUT-1)²⁹.

In accordance with the literature, a strong relation was found between the expression of HIF-1a and CA-IX^{11,13-15}. The relation with tumor hypoxia however is still not completely elucidated, since the expression of HIF-1a and CA-IX could not be correlated to needle electrode measurements^{13,19,24}. This might be explained by the fact that HIF-1a can be upregulated by other factors than hypoxia (e.g. nitric oxide, cytokines, oncogenes)¹⁹ and that HIF-1a sustainability and subsequent transactivation of target genes is dependent on the inhibition of two oxygen-dependent degradation pathways³⁵. In this study we included the analysis of OPN expression which is also upregulated during hypoxia but independently from main transcription factor HIF-1a and might therefore be of additional predictive value. In HNSCC, OPN expression has been associated with needle electrode measurements^{19,24}. In

Table 4. Patient and disease characteristics related to local recurrence after RT (local control) through Cox regression analysis in supraglottic LSCC.

Characteristic		Local recurrences per stratum	Univariate HR (95% CI)	p-value
HIF-1a expression	-Low -High	3/13 10/47	1 1.07 (0.29-3.87)	0.92
CA-IX expression	-Low -High	12/49 1/11	1 0.34 (0.04-2.58)	0.29
OPN expression	-Low -High	8/40 5/20	1 1.37 (0.45-4.17)	0.59
HIF-1a/CA-IX expression	-Both low -≥1 high	3/13 10/47	1 1.07 (0.29-3.87)	0.92
HIF-1a/CA-IX/ OPN expression	-All low -≥1 high	2/10 11/50	1 1.21 (0.27-5.45)	0.81
Gender	-Male -Female	11/43 2/17	1 0.38 (0.09-1.73)	0.21
Age (yrs)	-<64 -≥64	7/39 6/21	1 1.94 (0.65-5.80)	0.24
Primary symptom	-Hoarseness -Other	7/32 6/28	1 0.95 (0.32-2.83)	0.93
T-status	-1 -2	3/16 10/44	1 1.41 (0.39-5.11)	0.61
N-status	-0 -≥1	7/45 6/14	1 3.22 (1.08-9.62)	0.04
Hemoglobin	-Low -High	3/14 10/44	1 0.95 (0.26-3.46)	0.94

Table 5. Patient and disease characteristics related to death after RT (overall survival) through Cox regression supraglottic LSCC.

Characteristic		Local recurrences per stratum	Univariate HR (95% CI)	p-value
HIF-1a expression	-Low -High	4/13 22/47	1 1.23 (0.42-3.64)	0.71
CA-IX expression	-Low -High	21/49 5/11	1 0.83 (0.31-2.22)	0.70
OPN expression	-Low -High	16/40 10/20	1 0.99 (0.44-2.21)	0.98
HIF-1a/CA-IX expression	-Both low -≥1 high	4/13 22/47	1 1.23 (0.42-3.64)	0.71
HIF-1a/CA-IX/OPN expression	-All low -≥1 high	4/10 22/50	1 0.72 (0.24-2.17)	0.56
Gender	-Male -Female	20/43 6/17	1 0.69 (0.28-1.74)	0.43
Age (yrs)	-<64 -≥64	14/39 12/21	1 3.41 (1.50-7.71)	0.003
Primary symptom	-Hoarseness -Other	15/32 11/28	1 0.93 (0.43-2.02)	0.85
T-status	-1 -2	5/16 21/44	1 2.92 (1.06-8.09)	0.04
N-status	-0 -≥1	19/45 7/14	1 1.80 (0.74-4.38)	0.20
Hemoglobin	-Low -High	6/14 20/44	1 0.71 (0.28-1.81)	0.48

our study, co-expression of HIF-1a and OPN shows a slight trend. Literature is inconclusive on this as well. Interestingly, in an ovarian cancer cell line OPN has been shown to increase HIF-1a expression through the PI3-K/Akt pathway³⁶, which might be of influence in LSCC as well and might explain the weak association found in our study. The absence of an association between CA-IX and OPN expression has been reported before in HNSCC^{13,19,24}.

In the literature, expression of HIF-1a and CA-IX has been associated with impaired local control, locoregional control, overall survival and disease specific survival in predominantly advanced stage HNSCC treated with a variety of modalities^{11-13,15,19,24-27,37}, although contradictory results have been published as well¹²⁻¹⁶.

We previously demonstrated in a homogenous group of 91 patients with early-stage (T1-T2) LSCC primarily treated with RT and all selected from the glottic region that expression of HIF-1a and CA-IX was of prognostic value towards local control¹¹. In this study, we investigated the prognostic value of classical pre-treatment parameters and endogenous hypoxia markers HIF-1a, CA-IX and OPN in T1-T2 LSCC all located in the supraglottis selected from the same cohort of patients that were used to select the glottic LSCC¹¹. Our analysis of 60 supraglottic pre-treatment specimens revealed that neither HIF-1a nor CA-IX expression was found to be of prognostic value towards local control and overall survival (table 4 and 5). Also combining HIF-1a and CA-IX expression into a hypoxic profile (low HIF-1a and CA-IX expression vs. high HIF-1a and/or CA-IX) was of no prognostic value (figure 2a), suggesting that tumorhypoxia might not have as an important prognostic role in

supraglottic LSCC as it has in glottic LSCC.

Although the supraglottic LSCC demonstrated similar findings in terms of events during follow-up compared to our earlier findings in glottic LSCC¹¹ (table 2), there were differences as well. In the supraglottic LSCC significantly more women ($p=0.01$), more primary symptoms other than hoarseness ($p<0.001$), more T2-staged tumors ($p=0.002$) and more N+ cases were present ($p<0.001$)(table 1). These differences between glottic and supraglottic LSCC have been reported previously^{18,38}, suggesting that supraglottic and glottic LSCC might represent different entities.

Because we selected a very homogenous population of patients with supraglottic carcinoma primarily treated with radiotherapy only from our large cohort, the number of cases tested was relatively low and, therefore, our findings should be confirmed in a larger independent homogenous population study.

Several studies have shown that OPN expression is of prognostic value towards overall survival in HNSCC²¹⁻²⁴. However, in the present study, OPN expression demonstrated not to be of value in predicting overall survival and local control, confirming the findings of Nordmark et al¹³. Adding OPN expression to our hypoxic profile, created again 2 groups: low HIF-1a, CA-IX and OPN expression vs. high HIF-1a and/or CA-IX and/or OPN expression. OPN expression appeared to be of no additional value to our original hypoxic profile for both overall survival and local control.

In the literature, different findings in HIF-1a, CA-IX and OPN expression, its associations with clinical parameters and its prognostic value, could well be due to methodological aspects. Staining protocols and staining evaluation of HIF-1a, CA-IX and OPN expression show great diversity. Cut-off values used in the literature vary between 0-50% for predominantly nuclear HIF-1a staining^{12-15,17,24,25,37,39}, 0-50% for predominantly membranous CA-IX staining^{13,14,16,24,26,27} and 0-10% for cytoplasmatic OPN staining²¹⁻²⁴. We used cut-off values of 0.5%, 12.5% and 0.5% for HIF-1a, CA-IX and OPN expression respectively, based upon receiver operator characteristic curve analyses and taking in consideration the methods used by Schrijvers et al.

In our studies both local control and overall survival were used as clinical outcome parameters. In the literature there is a lack of uniformity in these parameters. However, for analyzing the predictive value of markers for success of primary treatment, the use of local control seems to be the most suitable alternative. Enabling comparison with other studies, we used overall survival as a clinical outcome parameter as well.

Finally, in the literature, study population characteristics differ considerably as well, with respect to T-stage, tumor location and treatment modality. Therefore, we investigated the prognostic value of hypoxia markers in a homogeneous group of T1-T2 supraglottic LSCC, treated with primarily RT, enabling a comparison with our earlier findings in glottic LSCC¹¹.

Interestingly, in the glottic LSCC the hypoxic profile was of strong prognostic significance towards local control and somewhat less to overall survival¹¹. These findings could not be confirmed in supraglottic LSCC, suggesting that supraglottic LSCC might represent a different biologic entity.

CONCLUSION

Although OPN expression seems to be related to HIF-1a expression, it was not found of additional prognostic value for local control in supraglottic LSCC.

Furthermore, neither HIF-1a, nor CA-IX, nor OPN was found to be of prognostic significance towards local control or overall survival in T1-T2 supraglottic LSCC primarily treated with RT. Our earlier findings in T1-T2 glottic LSCC¹¹ could not be confirmed in T1-T2 supraglottic LSCC. This inconsistency in results in these two homogeneous LSCC subgroups suggests that supraglottic carcinomas might represent a different biological entity compared to glottic LSCC.

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Assessment of hypoxic subvolumes in laryngeal cancer with ^{18}F - fluoroazomycinarabinoside (^{18}F -FAZA)-PET/CT scanning and immunohistochemistry

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ABSTRACT

Background and Purpose: 18F-fluoroazomycinaraboside (18F-FAZA) is a promising hypoxia radiopharmaceutical agent with outstanding biokinetic parameters. We aimed to determine the accuracy of 18F-FAZA-PET/CT scan in detecting hypoxic regions within the tumor using immunohistochemical markers in a pilot study.

Patients and methods: Eleven patients with primary or recurrent laryngeal squamous cell carcinoma were indicated for total laryngectomy (TLE). Patients underwent 18F-FAZA-PET/CT scan before TLE. Hypoxic regions inside the laryngeal tumor were determined. After TLE, regions with high uptake on 18F-FAZA-PET scan were selected for immunohistochemical examination for exogenous (pimonidazole) and endogenous (HIF1 α , CA-IX and GLUT-1) hypoxia markers. To assess the accuracy of 18F-FAZA-PET scanning, radiopharmaceutical accumulation was related with immunohistochemical expression of hypoxia markers.

Results: Inter- and intratumoral heterogeneity of tumor hypoxia was observed on 18F-FAZA-PET scan. Nine of the eleven tumors were hypoxic with 18F-FAZA-PET. Hypoxia could also be detected with pimonidazole, HIF1 α , CA-IX and GLUT-1 expression in some tumors. No clear association was observed between 18F-FAZA uptake and hypoxia markers.

Conclusions: This pilot study could not prove the accuracy of 18F-FAZA-PET in determining hypoxic subvolumes in laryngeal cancer. Further study is required to investigate the benefit of 18F-FAZA-PET imaging in radiotherapy planning.

INTRODUCTION

Tumor hypoxia is identified as a negative prognostic factor in patients with different solid tumors, including head and neck cancer.¹ It is associated with poor clinical outcome due to unfavorable biological characteristics, such as genetic instability, enhanced metastatic potential and invasiveness.² Hypoxic head and neck tumors treated with radiotherapy show a poor local control due to decreased radiosensitivity.^{3,4} Identification of tumor hypoxia may be useful, not only in prediction of outcome, but also in adjusting therapy to hypoxia in order to improve outcome.

Since the clinical relevance of tumor hypoxia became obvious, an increasing number of different detection methods have been reported.⁵ Understandably, metabolic imaging techniques, especially PET became important, using radiopharmaceuticals particularly developed for hypoxia visualization⁶, including 18F-labeled fluoromisonidazole (18F-FMISO), which so far is the most commonly used hypoxia tracer.⁷ The main disadvantage of 18F-FMISO is its relatively lipophilic properties, leading to low target-to-background ratio.⁸ More recently, hypoxia tracers with better kinetic features, like 18F-fluoroazomycin-arabinoside (18F-FAZA), have been developed. 18F-FAZA is less lipophilic than 18F-FMISO, increasing hypoxia-to-background ratio by a higher perfusion rate and a faster clearance from blood (reviewed by Halmos et al.⁶).

There are few studies comparing imaging techniques with immunohistochemical staining performed on biopsies from head and neck tumor to detect hypoxia (reviewed by Halmos et al.⁶). However, there is a lack of studies matching hypoxia in specific hypoxic subvolumes of whole tumor specimens using preoperative in vivo hypoxia imaging with immunohistochemistry on selected areas in the removed whole specimen. Whole specimen analysis is of great importance, because of the known heterogeneity of tumor hypoxia within the tumor mass.⁹ In the present pilot study we aimed to assess tumor hypoxic subvolumes in laryngeal cancer performing a preoperative 18F-FAZA-PET/CT scan and analyzing commonly used exogenous and endogenous hypoxia markers on selected areas of whole laryngectomy specimens. To determine whether 18F-FAZA-PET is suitable to define hypoxic subvolumes in the tumor, it is important to correlate 18F-FAZA accumulations with immunohistochemical expression of pimonidazole, HIF1 α , CA-IX and GLUT-1, which are considered representative markers for tumor hypoxia.⁵

MATERIALS AND METHODS

Patients

From February 2010 to June 2012, twelve adult patients with advanced or recurrent squamous cell carcinoma of the larynx with a tumor volume more than 2 cm³ were enrolled in this prospective pilot study. In all of these patients, total laryngectomy (TLE) was considered the treatment of choice according to our institutional protocols. The ethical committee of the University Medical Center Groningen (UMCG) approved the study protocol. All patients provided written informed consent before participation in the study. Patients underwent routine preoperative evaluation (e.g. blood tests, CT scan of the head and neck and the thorax, anesthesiology consult, maxillofacial- and radiotherapy consultation).

18F-FAZA-PET/CT

All patients underwent an 18F-FAZA-PET/CT scan one to five days prior to the scheduled TLE. Scans were performed at the Department of Nuclear Medicine and Molecular Imaging of the UMCG on a Biograph mCT 64 (Siemens Medical Solutions, Hoffman Estates, Knoxville, TN, USA) and executed according to EANM guidelines.¹⁰ Patients were injected with 370 MBq of 18F-FAZA intravenously approximately 120 minutes before scanning. A mid-thigh to the brain static PET acquisition was performed on the PET/CT tomography in a three-dimensional mode. The PET data were reconstructed with an iterative ordered subsets expectation maximization (OSEM), Time of Flight (TOF) and High Definition (HD) reconstruction algorithm with three iterations, 21 subsets with 8 mm Gaussian post-filter with a voxel size $2.04 \times 2.04 \times 2$ mm³. Scans were analyzed using the MIM Vista software (MIM corp., Version 6.1, Ohio, USA), a computer-based workstation for visualization, quantification, and analysis of PET/CT images.

Calculation of Tumor-to-Background (T/B) ratio

The tumor-to-background (T/B) ratio was determined along the following steps. First, the volume of interest (VOI) representing the gross tumor volume (GTV) on CT was created. The corresponding VOI was transferred to the PET image. The maximum standardized uptake value (SUVmax) was obtained by delineating the VOI comprising the entire tumor volume. A tumor free area in the neck muscle (sternocleidomastoid muscle) was chosen as a reference background. The mean SUV of this background area was calculated (SUVmean). Finally, the 18F-FAZA T/B ratio was assessed by calculating the ratio between SUVmax within the tumor and SUVmean background.

Immunohistochemistry

Approximately 120 minutes before TLE, 20 minutes, i.v. infusion of 500 mg/m² of pimonidazole (hypoxyprobe™-1, NPI inc.) dissolved in 100 ml 0.9% Sodium Chloride was administered. After removal of the resection specimen, the specimen was analyzed by the pathologist for clinical / histopathological diagnostics (pathologic signs and resection margins) as well as further evaluation in the context of the study.

At the department of pathology, the laryngectomy specimen was visually inspected and documented by taking regular photographs (example in Figure 1). The larynx specimen was cut open dorsally and then fixated with formalin for 24 hours (with a maximum of 72 hours). After fixation the specimen was laminated from caudal to cranial into slices of 3 mm. In cases of supraglottic tumors (case 4-11), one horizontal incision was made in the midline of the epiglottis. Next, the cranial part of the specimen was sliced vertically to achieve a better view on the tumor margins. Photo documentation of the slices was achieved as well (example in Figure 1). Guided by PET/CT, 1-2 biopsies were taken out of the region with highest 18F-FAZA accumulation and embedded in paraffin selected, for further immunohistochemistry. The remaining laryngeal tissue was not further analyzed in context of the study, but was prepared for routine histopathological examination.

All biopsies were stained with Hypoxyprobe™-1Mab1 (pimonidazole) mouse monoclonal antibody clone 4.3.11.3 (NPI inc., Burlington, Massachusetts, USA), HIF1α monoclonal mouse antibody clone 54 (BD Biosciences, Franklin Lakes, New Jersey, USA), CA-IX mouse monoclonal

antibody clone M75 (provided by Jaromir Pastorek, University of Bratislava, Slovakia) and GLUT-1 polyclonal Rabbit Anti-human antibody (DakoCytomation, Glostrup, Denmark). Different staining was performed on 4 µm paraffin sections. For HIF1α, CA-IX and GLUT-1 staining was performed as before.⁴ For Hypoxyprobe™-1Mab1 the following method was performed: slides were first deparaffinized in xylene for 10 minutes twice and then rehydrated through a series of ethanol dilutions and phosphate-buffered saline (PBS). Antigen retrieval was achieved with Protease 0.1% for 30 minutes at room temperature. To block endogenous peroxidase, 0.3% hydrogen peroxidase was applied for 30 minutes at room temperature. The slides were stained with the antibody against Hypoxyprobe™-1Mab1 (monoclonal mouse, 1:100) for 1 hour at room temperature. EnVision (DAKO, Glostrup, Denmark) for 30 minutes at room temperature was used as secondary antibody. Between all steps slides were washed three times with PBS. The peroxidase reaction was performed by applying 3,3'-diaminobenzide tetrachloride (DAB) for 10 minutes and slides were then washed with demineralised water. Finally the slides were counterstained for 2 minutes with hematoxylin and were dehydrated and fixated.

Scoring of staining

For HIF1α, CA-IX and GLUT-1 scoring was performed as described in a previous study.⁴ Scoring method for pimonidazole was set with an experienced pathologist based on previous studies.¹¹⁻¹⁶ Two separated teams scored all slides. Differences in results were resolved in a consensus meeting. Immunoreactivity was assessed in the nucleus above cytoplasmic background for pimonidazole and HIF1α and in the cell membrane for CA-IX and GLUT-1.^{4,11-16} Staining above the level of any cytoplasmic background was considered as positive staining. For HIF1α, CA-IX and GLUT-1 both percentage and intensity of positive tumor cells were scored, where intensity of positive staining was differentiated in weak and strong positive staining. The intensity for all three antibodies was relatively homogeneous and therefore not incorporated into the scoring data. For pimonidazole only the percentage of positive tumor cells was scored, regardless of intensity of staining.¹¹⁻¹⁶ In this way, for all four antibodies a percentage of positive stained tumor area relative to the total tumor area was obtained for each slide.

Statistical analysis

Patient characteristics, whole PET-T/B ratio and percentages of pimonidazole, HIF1α, CA-IX and GLUT-1 positive immunohistochemical staining were described.

To assess relation between 18F-FAZA accumulation and hypoxia markers, we performed scatter plot analysis and looked for visible trends in associations between them. Correlation significance analysis was not performed because of the diversity of data and small number of patients included in this pilot study.

All analyses were performed using Statistical Package for Social Sciences, version 22 (IBM SPSS Statistics 22).

RESULTS

Baseline characteristics

The study population consisted of 11 patients (10 males and 1 female) (details Table 1). One patient with recurrent T2N0M0 glottic laryngeal cancer was excluded, as the tumor volume was significantly smaller than 2 cm³ on histological evaluation but appeared larger on preoperative imaging due to radiotherapy-induced peritumoral edema.

The median age was 63 years (range 48–86). All patients had primary or recurrent squamous cell carcinoma of the larynx. Depending of tumor stage, size and localization, patients underwent a total laryngectomy alone, a total laryngectomy combined with ipsilateral or bilateral neck dissection of any kind or a total laryngectomy in combination with a neck dissection and a flap reconstruction. No adverse events were found after injection of 18F-FAZA or pimonidazole.

Imaging

The 18F-FAZA-PET/CT scan was performed 1–5 days (median 2 days) prior to the scheduled laryngectomy in all patients.

Visual analyses of the 18F-FAZA-PET scans showed clear uptake of 18F-FAZA in the tumor of nine out of the eleven patients. Using a tumor to background (T/B) threshold of $> 1.4^{17}$, in two patients 18F-FAZA uptake remained below the threshold. The other nine patients showed an uptake of 18F-FAZA over 1.4 (for details see Table 1.). Of the nine patients with positive 18F-FAZA uptake, five tumors showed a rather homogeneous 18F-FAZA uptake across the whole tumor. In four patients a more heterogeneous uptake of 18F-FAZA was observed. In the cases of heterogeneous uptake, the pattern showed a high 18F-FAZA uptake central in the tumor and a lower uptake in the peripheral tumor margins. The median 18F-FAZA T/B ratio was 1.6 (range: 1.2 – 2.5).

Immunohistochemistry

All biopsies showed positive immunostaining for pimonidazole in 15% to 95% of tumor cells. Only 6 biopsies showed positive staining for CA-IX with 4–53% of positive tumor cells. All biopsies showed positive immunostaining for GLUT-1 varying between 13% and 85% of tumor cells. In one of the biopsies, prepared for HIF1 α staining, no tumor was left. Therefore only biopsies of ten patients were analyzed. Of these, 8 patients had a positive immunostaining varying between 5% and 100% of tumor cells.

Photomicrographs in Figure 1 are representative of the immunostaining patterns of pimonidazole, HIF1 α , CA-IX and GLUT-1.

Relation between PET and Immunohistochemistry

The relation between 18F-FAZA uptake (expressed in T/B ratio) and immunohistochemical expression of the different hypoxia markers (expressed in % of staining) is shown in Figure 2. No obvious trends between the 18F-FAZA uptake and any of the hypoxic markers are seen on the scatter plot diagrams.

Table 1. Patient characteristics, 18F-FAZA-PET and immunohistochemical data.

Pt.	Gen-der	Stage (original)	Subsite	Indication TLE	Type of surgery	Duration of surgery (hours)	Adjuvant therapy	Macroscopic tumor diameter (mm)	Differen-tiation	Angio-in-vasion	Follow-up time (months)/ status	Back-ground PET	Whole PET T/B	Visual PET +/-	Uptake type	Pimo %	HIF1a %	CAIX %	GLUT-1 %
1	M	T ₁ N ₀ M ₀	Supraglottic	Recurrence	TLE + SNDRL	7.5	No	30	Poorly	No	52/NED	1.10	1.50	+	Homo-geneous	80	0	11	85
2	M	T ₄ N ₁ M ₀	Glottic	Advanced tumor	TLE + SNDRL	8.0	RT	35	Moderately	No	52/AWD	1.10	1.70	+	Hetero-geneous	85	10	4	18
3	M	T ₄ N ₁ M ₀	Supraglottic	Advanced tumor	TLE + MRNDLR	9.5	RT + CT	70	Moderately	Yes	45/NED	1.25	1.68	+	Hetero-geneous	16	45	0	50
4	M	T ₃ N ₀ M ₀	Supraglottic	Recurrence	TLE	4.5	No	14	Moderately	No	36/NED	1.35	1.50	+	Homo-geneous	95	0	0	13
5	M	T ₁ aN ₀ M ₀	Glottic	Recurrence	TLE	4.5	RT	35	Moderately	No	23/DOD	1.10	1.80	+	Homo-geneous	75	5	0	35
6	M	T ₁ N ₂ aM ₀	Supraglottic	Recurrence	TLE + SNDRL	12.0	RT	42	Poorly	Yes	5/DOD	1.10	1.20	-	Homo-geneous	60	28	35	50
7	M	T ₄ N ₀ M ₀	Glottic	Advanced tumor	TLE + SNDRL + PM flap	13.0	RT	36	Moderately	Yes	27/NED	1.40	1.20	-	Homo-geneous	50	100	0	13
8	M	T ₄ N ₂ bM ₀	Supraglottic	Advanced tumor	TLE + MRNDR + SNDL	11.5	RT	35	Moderately	Yes	29/NED	1.35	2.00	+	Hetero-geneous	15	65	18	50
9	M	T ₃ N ₀ M ₀	Supraglottic	Recurrence	TLE	7.5	No	15	Moderately	No	22/NED	1.25	2.50	+	Homo-geneous	21	53	53	78
10	F	T3N ₀ M ₀	Supraglottic	Advanced tumor	TLE + MRNDL + SNDL + RFFF	10.5	RT	32	Moderately	No	24/NED	1.10	1.45	+	Hetero-geneous	70		0	80
11	M	T ₄ N ₂ aM ₀	Supraglottic	Advanced tumor	TLE + MRNDR + SNDL	9.0	RT + CT	40	Moderately	No	24/DOD	1.23	1.60	+	Homo-geneous	80	53	8	60

Pt, patient; TLE, total laryngectomy; SND, selective neck dissection; RL, refers to side, right, left; MRND, modified radical neck dissection; PM flap, pectoralis major flap; RFFF, radial forearm free flap; RT, radiotherapy; CT, chemotherapy; DOD, dead of disease; NED, no evidence of disease; AWD, alive with disease; T/B, tumor-to-background ratio; pimo, pimonidazole.

Figure 1. 18F-FAZA-PET/CT, TLE specimen and immunohistochemical slides from patient 1.

Figure 2. Scatterplot in which 18F-FAZA uptake (expressed in T/B ratio) is plotted against immunohistochemical expression assigned as percentage of positive immunostained tumor cells relative to the total tumor area.

DISCUSSION

This is the first study attempting to assess hypoxia subvolumes with 18F-FAZA-PET and immunohistochemical markers in patients. In the present study significant inter- and intratumoral heterogeneity of tumor hypoxia was observed using 18F-FAZA-PET in laryngeal cancer. Using a cut-off of 1.4 T/B ratio for hypoxic tumor¹⁷, two tumors were found to be not hypoxic with a T/B ratio of 1.2, one tumor was found to be only marginally hypoxic with a T/B ratio of 1.45, the other 8 tumors found to be hypoxic with a maximum T/B of 2.5. After total laryngectomy the same laryngeal tumor specimens were tested for hypoxia using well-defined exogenous and endogenous hypoxia markers. Positive immunohistochemical staining was found within nearly all hypoxic areas, detected by 18F-FAZA-PET. However, there was no clear association between the 18F-FAZA-PET uptake values and the quantitative expression of immunohistochemical hypoxia markers suggesting that 18F-FAZA uptake may reflect tumor hypoxia, but not necessarily correlate with the spatial distribution of the extent of hypoxia. From our previous experience we know that tumor hypoxia is a dynamic process due to constantly changing tumor micro environment such as differences in dynamic blood flow and chaotic blood supply.¹⁸ Therefore, tumor cells that are hypoxic today may or may not be hypoxic

at subsequent time point. Hence, it is cumbersome to compare immunohistochemistry results with that of 18F-FAZA-PET. Especially when there is a gap of 2-5 days in between PET and TLE.

The 18F-FAZA-PET results of the present study are in line with other studies. The T/B ratio values are found to be relatively low, compared to other tumor sites, but similar with the values of other studies performed on head and neck cancer. Postema et al. found comparable T/B ratio values (1.2-2.7) in nine head and neck cancers.¹⁹ In the first published 18F-FAZA-PET study on humans showed different intratumoral spatial distribution in head and neck cancers, varying between a single area, multiple diffuse areas and no area of 18F-FAZA uptake in the tumor²⁰, similar results were found as in the present study.

We found 18F-FAZA uptake in nine out of eleven patients, comparable to Grosu et al. (15/18).²⁰ However, real comparison of 18F-FAZA uptake data is hampered as different studies apply different settings. Another important aspect is tumor site; using 18F-FAZA-PET, high-grade gliomas are found to be highly hypoxic, while lymphomas are less hypoxic¹⁹ and no relevant hypoxia could be detected in prostate cancer.²¹ To overcome the problem of different definitions of hypoxia, a recent study suggested selecting the threshold upon non-hypoxic normal tissue.¹⁷ Based on the neck muscle uptake of 40 patients, the T/M value of 1.4 and above was established as hypoxic.

The findings of the present study contradict the results of 18F-FAZA-PET studies on animal model, where accurate quantitative hypoxia maps were generated.²² In that study high correlation was found between 18F-FAZA accumulation in autoradiograms and immunofluorescence images with pimonidazole staining using pixel-by-pixel comparison.

In studies, concerning other radiolabeled nitroimidazoles, Troost et al also found a significant correlation between 18F-FMISO-PET imaging and pimonidazole in head and neck cancer.²³ However, in another validation study only a weak correlation was found between 18F-FMISO uptake and pO_2 histography, most likely due to heterogeneity in intratumoral hypoxia.²⁴ Theoretically, geometrical mismatch can also be responsible for the lack of correlation. Ideally, the whole specimen should have been co-registered by 3D matching together with the scan, like microscopic autoradiography or for instance in the study of Daisne et al.²⁵ However, this would have interfered with routine histopathological examination of the specimen for clinico-pathological correlation. The histological processing of the removed tissue was performed according to the standard guidelines, as tumor features, like differentiation grade, perineural growth, cartilage invasion, surgical margins had to be evaluated because of the clinical consequences. Instead of processing the whole specimen, biopsies were taken from for hypoxia suspected areas on the 18F-FAZA-PET. We chose explicitly the larynx for this pilot study, as its cartilaginous skeleton could serve as reference. On the other hand, the experimental nature of the mentioned study²² could hardly or never be reproduced in human head and neck cancer patients. Such a pixel-by-pixel accurate spatial hypoxia assessment does not seem to be obtainable due to patient and tumor factors, like peritumoral edema, tumor necrosis, and motion artifacts. Interestingly, the same study²² also found a controversial result with respect to the endogenous marker GLUT-1. Difficulties in the detection of hypoxia in patients using PET scan have already been implied by other authors.²⁶ The slow tracer retention in hypoxic areas and the slow clearance of tracers from non-hypoxic tissue necessitate to average signal over large areas; therefore the resolution of hypoxia targeted PET is still not accurate enough.

The lack of a clear association between hypoxic areas detected by 18F-FAZA-PET and immunohistochemical staining with endogenous markers can also be explained by acute blood perfusion changes during the surgery in comparison to the PET scan thus changing oxygenation grade. Acute decrease in blood perfusion in otherwise non-hypoxic tumor areas can lead to 18F-FAZA accumulation, while the endogenous markers are not present and the other way around: recently reoxygenated, otherwise hypoxic areas can be negative by 18F-FAZA while the hypoxia markers are still present. This has been earlier implicated in case of CA-IX mismatch.²⁷ Other explanation is the low specificity of endogenous markers to hypoxia. For instance, HIF1 α , CA-IX and GLUT-1 accumulation can be observed under other conditions than hypoxia, e.g. hypoglycemia and acidosis.²⁸ The plasma half-life of pimonidazole is 5.1 ± 0.8 hours.²⁹ Since most surgical procedures in this study took more than the plasma half-life (median 9 hours (range 4.5-13 hours) this might have influenced the immunohistochemical expression. However, in this study no correlation could be observed between pimonidazole expression and the duration of surgery, suggesting operation time did not have influenced pimonidazole expression neither by clearance of pimonidazole out of the body or by devascularisation of tumor during operation.

Both primary and recurrent laryngeal cancer patients have been included in the study. Although earlier radiotherapy could have had impact on tumor hypoxia in the recurrent tumors, there has been no differences seen neither in the 18F-FAZA uptake, nor in the expression of hypoxia markers between the primary and recurrent cases. The aim of the study was to investigate hypoxia regardless to the etiology of hypoxia. Thus, if some of the hypoxia cases in the recurrent group were radiation-induced, it does not influence our results.

The traditional SUVmax value or T/B ratio may not reflect the changes in global tumor microenvironment. They are characteristics of the single voxel with the least oxygenation status within the tumor. It is unlikely that single hypoxic voxel measurement within the tumor reflect the oxygenation status of the entire tumor volume. Hence, further studies should incorporate voxel-by-voxel analysis, which provides detailed information about the hypoxic distribution across the entire tumor rather than a single voxel. This will bring us more understanding of tumor biology and characterize the tumor heterogeneity.

Based on the present pilot study, it cannot be confirmed that 18F-FAZA-PET is suitable to detect the spatial distribution of tumor hypoxia in laryngeal squamous cell carcinoma. Although there was expression of hypoxia markers in all tumors and 18F-FAZA accumulations in most tumors, both suggesting hypoxic tumors, no clear association could be found between them. Therefore, based on the present pilot study, we conclude that 18F-FAZA is insufficiently validated to be used in hypoxia guided radiotherapy dose escalation protocols. Further studies are required to confirm 18F-FAZA as a marker for the extent of hypoxia and its use in everyday clinical practice.

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Phosphorylated FADD is not prognostic for local control in T1-T2 supraglottic laryngeal carcinoma treated with radiotherapy

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ABSTRACT

Objective: The FADD gene is located in the chromosome 11q13-region and is frequently amplified in head and neck squamous cell carcinoma. Expression of FADD and its phosphorylated isoform (pFADD) have been associated with aggressive tumor growth, lymph node metastasis and overall survival. Previously, we demonstrated that pFADD expression was related to a significantly improved local control in early stage (T1-T2) glottic laryngeal squamous cell carcinoma (LSCC). The aim of this study was to examine the prognostic value of pFADD and FADD in T1-T2 supraglottic LSCC treated with primarily radiotherapy.

Methods: Tumor tissue sections of 60 patients with T1-T2 supraglottic LSCC treated with primarily radiotherapy were assessed immunohistochemically for expression of pFADD and FADD. Expression percentages and clinical parameters and their associations with clinical outcome were studied, using Cox regression and Kaplan-Meier survival analyses. Expression percentages in supraglottic and glottic LSCC were compared using the Mann-Whitney U test.

Results: Expression of pFADD and FADD in supraglottic and glottic LSCC did not significantly differ. In supraglottic LSCC both pFADD and FADD did not show prognostic value for local control (HR1.00, 95%CI 0.98-1.03; HR1.03, 95%CI 0.60-1.78, respectively) and overall survival (HR0.99, 95%CI 0.98-1.01; HR1.19, 95%CI 0.83-1.71 respectively). In this cohort, lymph node status was the best predictor for local control (HR3.73, 95%CI 1.30-10.67).

Conclusion: In this homogeneous cohort of T1-T2 supraglottic LSCC primarily treated with radiotherapy, lymph node status was associated with local recurrence whereas the expression of pFADD was not.

INTRODUCTION

Chromosomal DNA amplification has been reported in various human malignancies and is generally accepted as a mechanism resulting in increased gene expression.¹ This implicates a selective advantage for cells containing increased mRNA levels for genes found within the amplified region. In head and neck squamous cell carcinoma (HNSCC), amplification of the 11q13.3 chromosome region occurs frequently (36%)^{2,3} and has been correlated with aggressive tumor growth, lymph node metastasis, decreased locoregional control and overall survival (OS).⁴⁻⁹

In HNSCC the commonly amplified region contains 9 genes which are overexpressed when amplified: FADD (Fas-associated death domain), CCND1, TPCN2, PPFIA1, FLJ42258, CTTN, FGF19, ORAOV1 en ANO1.⁷ A comprehensive analysis of gene amplification and expression levels revealed that of all genes in the 11q13 amplicon in laryngeal squamous cell carcinoma (LSCC), FADD was amplified and overexpressed most frequently. Furthermore, FADD amplification correlated well with increased FADD protein expression in LSCC, suggesting that FADD is a driving gene in the 11q13 amplicon.⁷

The classical function of FADD is to mediate apoptosis. However, several reports demonstrated that FADD is involved in cell cycle regulation as well.¹⁰ FADD can be phosphorylated (pFADD) at a specific serine residue (SER194), which is associated with nuclear localization, predominantly in the G2/M phase of the cell cycle.¹¹ This suggests that phosphorylation of FADD and not expression of wild-type FADD is involved in cell cycle regulation and cell proliferation.

In 167 advanced stage LSCC, treated primarily with surgery, radiotherapy or a combination of both modalities, we previously demonstrated that increased protein expression of FADD and pFADD was associated with worse disease specific survival.⁷ In advanced stage oral squamous cell carcinoma, treated with surgery and postoperative radiotherapy, high pFADD expression was associated with an improved local control (LC) (Pattje et al., submitted for publication). And previously we reported, using a homogeneous study population of 92 patients with early stage (T1-T2) glottic LSCC all treated with radiotherapy only, high pFADD levels in 67% of the cases. In this cohort 18% of the cases developed local recurrent disease. High levels of pFADD were associated with a significantly better LC, independent of non-phosphorylated FADD levels, suggesting that expression of pFADD might mediate the sensitivity of neoplastic cells to radiotherapy.¹² In agreement with these observations, we showed that in HEK293 cells, transfected with a phosphorylated mimicking FADD variant, revealed an increased radiosensitivity determined by the *in vitro* cell survival assay compared to cells expressing the non-phosphorylated mimicking FADD isoform (Pattje et al, submitted for publication).

HNSCC comprises a wide spectrum of neoplasms with multiple involved subsites, each characterized by different patterns of clinicopathological behavior, risks and presumably tumor biology, suggesting that the various subsites should be considered as different entities and thus need distinctive therapeutic strategies.^{13,14} For instance, in the larynx, supraglottic LSCC is considered another entity as those originating from the glottis, based on epidemiology,¹⁵ invasive/metastatic potential¹⁶ and lymph drainage.¹⁷

We previously investigated the role of (p)FADD expression as a predictor for LC in a homogeneous

cohort of glottic LSCC.¹² The goal of this study was to investigate the prognostic value of pFADD and FADD expression in a comparable homogeneous group of 60 pretreatment biopsies of T1-T2 supraglottic LSCC, treated with primarily radiotherapy with curative intent. Association of (p)FADD expression with LC and OS was investigated. In addition, the association with classical prognostic markers of clinical outcome such as gender, age, primary complaint, T-status, N-status and pre-treatment hemoglobin level were investigated. These data were compared to previous data in glottic LSCC.

MATERIAL AND METHODS

Patients

The supraglottic as well as the glottic LSCC cohort of patients, used in this study, was described in detail previously.^{18,19} Between 1990 and 2008, 1286 patients were diagnosed with and/or treated for a laryngeal malignancy in the University Medical Center Groningen. Using the patient charts, a database was constructed retrospectively, gathering clinical, histopathological and follow-up data. Collection of patient data and tissue samples was approved by the Medical Ethics Committee of our hospital and written informed consent was obtained from patients included in this study.

For this study a selection of patients from this database was included using the following criteria: histologically confirmed squamous cell carcinoma, located in the larynx, clinically staged T1 or T2, treated with radiotherapy with curative intent, without any other previous treatment.

Ultimately, 247 patients with T1-T2 LSCC were retrieved in our database. Because of second primary tumors, previous chemo- or regional radiotherapy, ten patients were excluded. Of all remaining patients formalin-fixed, paraffin-embedded pre-treatment biopsies were obtained, revised by an experienced pathologist. From 152 patients sufficient tumor tissue was available of which 92 were of glottic and 60 of supraglottic origin.

A summary of the pre-treatment characteristics is demonstrated in table 1. In general, HNSCC patients in our institution are clinically evaluated every 3 months the first and the second year and every 6 months the third, fourth and fifth year after completing radiotherapy.

Of 60 supraglottic LSCC patients, 14 developed a local recurrence. Nine patients were treated with a total laryngectomy with/without neck dissection. Five patients received palliative or no further treatment. Thirty-two patients died, of which 11 died of disease. All follow-up data are presented in table 2.

Treatment

As reported previously, all patients in this cohort were treated primarily with exclusively radiotherapy, using 6MV linear accelerator equipment.^{18,19} Before the year 2000, planning of field arrangements was performed by direct simulation and from 2000 onwards by using contrast-enhanced CT-scans.

In patients with T1-T2aNO LSCC, clinical target volume consisted of the gross tumor volume with a 1cm margin. Direct simulation was achieved, using the inferior border of the hyoid bone and the cricoid cartilage as field borders. In this group of patients two opposing lateral fields were used with a median fraction dose of 2.0Gy (5 fractions/week) up to 66-70Gy in 7 weeks.

Table 1. Pretreatment clinical and immunohistochemical characteristics (n=60).

Parameter	Subgroup	Supraglottis	pFADD expr.			FADD expr.		
		n (%)	Low expr. n (%)	High expr. n (%)	p- value	Low expr. n (%)	High expr. n (%)	p- value
Gender	-Male	44 (73.3)	12 (27.3)	32 (72.7)	0.76	33 (78.6)	9 (21.4)	0.82
	-Female	16 (26.7)	5 (31.3)	11 (68.8)		13 (81.3)	3 (18.8)	
Age (years)	-Median (Range)	62.0 (33-96)			0.65			0.34
	-Mean	62.8						
	-≤ 64	38/60 (63.3)	10 (26.3)	28 (73.3)		30 (83.3)	6 (16.7)	
	->64	22/60 (36.7)	7 (31.8)	15 (68.2)		16 (72.7)	6 (27.3)	
T-status	-1	16/60 (26.7)	6 (37.5)	10 (62.5)	0.35	12 (85.7)	2 (14.3)	0.50
	-2	44/60 (73.3)	11 (25.0)	33 (75.0)		34 (77.3)	10 (22.7)	
N-status	-0	45/59 (76.3)	13 (28.9)	32 (71.1)	0.98	36 (83.7)	7 (16.3)	0.32
	->0	14/59 (23.7)	4 (28.6)	10 (71.4)		10 (71.4)	4 (28.6)	
	-1	9/59 (15.3)						
	-2	4/59 (6.8)						
	-3	1/59 (1.7)						
	-x	1/60						
Hemo-globin (mmol/l)	-Median (Range)	9.0 (6.7-15.7)			0.44			0.88
	-Mean	9.3						
	-Low	14/58 (24.1)	5 (35.7)	9 (64.3)		10 (76.9)	3 (23.1)	
	-High	44/58 (75.9)	11 (25.0)	33 (75.0)		34 (79.1)	9 (20.9)	
	-Unknown	2/60						
pFADD	-Median (Range)	80 (5-100)	Staining %					
	-Mean	75.2						
	-Low	17/60 (28.3)						
	-High	43/60 (71.7)						
FADD	-Median (Range)	2 (0-4)	Stain. int.					
	-Mean	1.97						
	-Low	40/60 (66.7)	Staining %					
	-High	20/60 (33.3)						
	-Median (Range)	0 (0-100)						
	-Mean	20.2						

In the remaining T2 LSCC patients or in case of lymph node metastasis, the initial clinical target volume consisted of the primary tumor, the pathological lymph nodes with a 1cm margin and bilateral elective nodal areas. For both the boost volume, consisting of the primary tumor and the pathological lymph nodes, as well as for the planning target volume, a margin of 0.5cm was used. Two opposing lateral fields were used for the site of the primary tumor and the lymph nodes located in the upper neck levels. An anterior field for the lower neck levels was only added, in the case of anatomical variations. To the initial planning target volume a total dose of 46Gy was delivered. Then a boost dose of 24Gy was delivered to the primary tumor and pathological lymph nodes (70Gy in total).

In general, most patients received conventionally fractionated radiotherapy. However, from 2000 onwards, patients generally received accelerated fractionated radiotherapy (5 fractions/week with additionally a second fraction on Friday afternoon, to a total dose of 70Gy in 6 weeks).

Table 2. Follow-up data.

Follow-up parameter	Subspecification	Supraglottis n=60 n (%)
Events in follow-up	-Patients with any event -Local recurrence -Regional recurrence -Distant recurrence (solitary) -2nd primary head/neck region -Death Death of disease Death not of disease Unknown	35/60 (58.3) 14/60 (23.3) 1/60 (1.7) 2/60 (3.3) 1/60 (1.7) 32/60 (53.3) 11/32 (34.4) 18/32 (56.3) 3/32 (9.4)
Time to local recurrence (months)	-Median (Range) -Mean	13.5 (5-18) 11.7
Time to loc./reg. recurrence (months)	-Median (Range) -Mean	14.0 (5-57) 17.4
Time to loc./reg./dist. recurrence (months)	-Median (Range) -Mean	14.0 (5-57) 17.4
Time to death (months)	-Median (Range) -Mean	38.0 (6-154) 48.3
Time to last follow up (months)	-Median (Range) -Mean	54.5 (5-169) 56.7

Immunohistochemistry

For immunohistochemical staining 4µm serial sections were cut. The sections were deparaffinized in xylene twice for 10 minutes and rehydrated through a series of decreasing ethanol dilutions and phosphate buffered saline (PBS). To verify histology hematoxylin and eosin staining was performed.

Details on the immunohistochemical stainings were reported previously.¹² Briefly, antigen retrieval was achieved by heating in a microwave in preheated EDTA buffer (pH 8.0) for 15 minutes at 100°C for pFADD or by incubation overnight in Tris-HCl (pH 9.0) at 80°C for FADD. To block endogenous peroxidase activity, 0.3% hydrogen peroxide was applied for 30 minutes at room temperature. The slides were then washed three times with PBS. For FADD immunostaining the sections were incubated with the primary antibody (A66-2, BD Biosciences, New Jersey, USA) diluted 1:100 for 60 minutes at room temperature, followed by the secondary (1:100, RAMPo, Dakocytomation) for 30 minutes at room temperature and tertiary antibody (1:100, GARpo, Dakocytomation) for 30 minutes at room temperature. For pFADD immunostaining, sections were incubated with the primary rabbit polyclonal antibody (Ser194 pFADD, BD Biosciences, New Jersey, USA) diluted 1:25 overnight at 4°C followed by Envision treatment (Dako, Glostrup, Denmark) for 30 minutes at room temperature. Subsequently, the peroxidase reaction was performed by applying 3,3'-diaminobenzide tetrachloride for 10 minutes. Finally, after washing the slides with PBS, they were counterstained with hematoxylin for 2 minutes, dehydrated and mounted.

Evaluation of immunohistochemical staining

The evaluation of immunohistochemical staining was identical to our earlier publications.^{8,12} Both the percentage of positive neoplastic cells as well as the intensity of the expression (-, +/-, ++, +++) were evaluated. For FADD, only staining intensity was used for clinical evaluation. As previously reported, in order to obtain a binary distribution the following groups were defined: low (-, +/-, +) and high expression (++, +++).¹² For clinical evaluation of the immunostaining of pFADD, the percentage of neoplastic cells with any nuclear staining (+/-, ++, +++) was determined. The same cut-off value of 71% of neoplastic cells with pFADD expression that was previously determined by Receiver Operating Curve (ROC) analyses in relation to local control, was used. Percentages of positive staining above the cut-off level were considered as high expression, and those below as low expression.¹² Both blinded for clinical outcome, 2 observers evaluated all slides independently. During conference microscope sessions, occasional discordant scorings were evaluated and resolved.

Statistical analysis

The Spearman's rank correlation analysis was performed to evaluate associations between the expression parameters. The relations between expression percentages and clinical characteristics were evaluated using logistic regression analysis. The Mann-Whitney U test was used in order to compare expression parameters in supraglottic and glottic LSCC.

Local recurrence was defined as tumor recurrence at the primary tumor site. Time calculations were performed, using the date of diagnosis as a starting point and the date of local recurrence, death or last follow-up visit as an endpoint.

Both Kaplan-Meier analysis, log-rank test and univariate Cox proportional hazard modeling was used to assess which tumor variables were of predictive value towards local recurrence and/or death. A p-value of ≤ 0.05 was considered to be statistically significant. The analyses were performed using identical cut off definitions, reported previously in glottic LSCC.¹² All statistical analyses were performed using the SPSS 20.0 software (Somers, USA).

RESULTS

Expression of FADD and pFADD in supraglottic LSCC

High FADD expression was found in 20/60 cases (33%) and high pFADD expression was found in 43/60 cases (71.7%)(table 1). Both pFADD and FADD immuno stainings were heterogeneously distributed within the tumor tissue. Furthermore, pFADD staining was mainly found in the nucleus, while FADD staining in the cytoplasm, but in the nucleus as well (fig. 1a-b). The Spearman's correlation test demonstrated that pFADD expression percentages did not correlate with FADD expression percentages ($r=0.049$, $p=0.71$) and intensity ($r=0.128$, $p=0.33$).

Figure 1. Positive staining for pFADD (A) and FADD (B) in supraglottic laryngeal squamous cell carcinoma. Original magnification, 200x.

Expression of pFADD and FADD is not associated with clinicopathological features and clinical outcome in T1-T2 supraglottic LSCC

Using logistic regression, no significant associations were found between pFADD and FADD expression parameters and clinical characteristics such as gender, age, T-stage, N-stage and hemoglobin level (table 1). Kaplan Meier and Cox regression analysis revealed that both expression of pFADD (HR1.00, 95%CI 0.98-1.03) and FADD (HR1.03, 95%CI 0.60-1.78) were not associated with LC (fig 2A-B, table 3). The expression of pFADD and FADD was not related to OS in both Kaplan Meier and Cox regression analysis (HR0.99, 95%CI 0.98-1.01; HR1.19, 95%CI 0.83-1.71 resp.) (fig. 3A-B, table 3).

Table 3. Patient and disease characteristics related to local recurrence (local control) and death (overall survival) after RT through Cox regression analysis in supraglottic LSCC.

	Local control		Overall survival	
Characteristic	Univariate HR (95% CI)	p-value	Univariate HR (95% CI)	p-value
pFADD expression	1 1.00 (0.98-1.03)	0.58	1 0.99 (0.98-1.01)	0.26
FADD expression	1 1.03 (0.60-1.78)	0.90	1 1.19 (0.83-1.71)	0.33
Gender	1 0.41 (0.09-1.85)	0.25	1 1.12 (0.50-2.47)	0.79
Age (yrs)	1 1.59 (0.55-4.58)	0.40	1 3.31 (1.59-6.89)	0.00
T-status	1 1.45 (0.41-5.21)	0.57	1 2.08 (0.87-4.95)	0.10
N-status	1 3.73 (1.30-10.67)	0.01	1 2.39 (1.13-5.07)	0.02
Hemoglobin	1 1.02 (0.97-1.03)	0.89	1 0.99 (0.97-1.01)	0.22

Seven out of 14 cases with lymph node positivity developed a local recurrence after radiotherapy compared to 7/45 cases with no lymph node involvement. Univariate Cox regression revealed that N-status is associated with LC (HR3.73, 95%CI 1.30-10.67) and both N-status and age with OS (HR2.39, 95%CI 1.13-5.07; HR3.31, 95%CI 1.59-6.89 resp.)(table 3).

Figure 3. KM-analysis for pFADD (a) and FADD (b) on death.

Expression of pFADD and FADD is similar in supraglottic and glottic T1-T2 LSCCs

In order to compare expression percentages in 60 T1-T2 supraglottic (present study) and in 85 T1-T2 glottic LSCC¹², the Mann Whitney U test revealed no statistical significant differences for pFADD or FADD expression ($p=0.58$, $p=0.45$ respectively). Since FADD expression has been defined as maximum expression intensity only in other studies, maximum intensities for FADD expression were compared as well.^{8,12} No significant differences could be found ($p=0.93$)(table 4).

Table 4. Expression characteristics for pFADD and FADD in glottic and supraglottic LSCC.

		Supraglottis	Glottis	p-value (Mann-Whitney U Test)
pFADD expression percentage	Median (Range)	80 (5-100)	80 (20-100)	0.58
FADD expression percentage	Median (Range)	0 (0-100)	0 (0-100)	0.45
FADD intensity	Median (Range)	2 (0-4)	2 (0-4)	0.93

DISCUSSION

Annually 650.000 new cases of HNSCC are diagnosed worldwide of which 23% is located in the larynx.²⁰ In early stage supraglottic LSCC radiotherapy is the main treatment modality. However, in the literature LC rates are approximately 68% and 54-60% for T1 and T2 supraglottic LSCC respectively.^{21,22} In case of radiotherapy failure or recurrent disease, salvage surgery through total laryngectomy is obligatory, which is accompanied by high rates of morbidity and mortality.

Currently, prediction of radiotherapy failure is based upon TNM-staging, which is of low predictive value. Therefore, there is a need for new prognostic markers to predict therapeutic outcome.

The relation between pFADD and FADD expression and their functions have not yet been fully elucidated. The classical function of FADD is to mediate Fas-induced apoptosis by recruiting caspase-8 and -10 to form the death-inducing signal complex, thereby activating the cysteine protease cascade and ultimately inducing apoptosis.²³ In addition, pFADD has been implicated in cell cycle regulation.¹⁰ At the G2/M transition of the cell cycle, FADD is phosphorylated by casein kinase (CK)1 α at a specific residue (SER194).¹¹ Moreover, pFADD and CK1 α were shown to co-locate in the nucleus.

Recently, we demonstrated in a group of 111 patients with T1-T4 oral squamous cell carcinoma treated with primary surgery and postoperative radiotherapy that high expression of pFADD was associated with a better LC. Moreover, in vitro experiments in HEK293 cells showed an increased radiosensitivity in cells that overexpressed pFADD, all together implicating a key role for pFADD in cell cycle control (Pattje et al., submitted for publication).

The association between (p)FADD expression and clinical outcome in HNSCC is not always significant.^{8,12,24} We demonstrated that in a group of 167 T1-T4/N0-N3 LSCC treated with surgery with or without radiotherapy, FADD and pFADD protein expression was related to a worse disease specific survival, using univariate Cox regression analysis.^{7,8} Because of the heterogeneity of this cohort regarding tumor stage, nodal stage and treatment modality, no firm conclusion could be drawn with respect to the prognostic value of these markers towards radiotherapy. Hence, we investigated the prognostic value of pFADD and FADD in a homogeneous cohort of 92 T1-T2/N0-N1 glottic laryngeal carcinomas treated with radiotherapy only. High expression of pFADD was associated with significantly better LC. High expression of FADD showed a trend towards improved LC.¹²

From the same large database at the UMCG used to select glottic LSCC, supraglottic LSCC were selected and in this homogeneous cohort expression of pFADD was not related to LC. The strength of our study is the homogeneity of the population under study. Despite the relatively small sample size, our cohort represents one of the largest reported homogeneous populations of early stage supraglottic LSCC curatively treated with radiotherapy only and the fact that our data could be compared directly to a similar population with glottic LSCC. Many possible confounding factors such as immunohistochemistry, scoring methods and treatment modalities are identical in both the earlier glottis and this supraglottic study. Moreover, the tissue-processing and expression analysis were done in the same laboratory and all patients with LSCC were treated by the same multidisciplinary Head and Neck team. A weak point of the present study is that despite the homogeneity of both cohorts, the numbers of supraglottic LSCC are relatively small. Therefore confirmation of our data by others in independent cohorts is needed.

Few studies compared the association between expression levels of other biomarkers with clinical outcome in both glottic and supraglottic LSCC. Goulioumis et al. demonstrated that expression of ILK, p-AKT, AR, ER-B, E-cadherin and vimentin was not, but expression of B-catenin was distinct.²⁵ Kourelis et al. reported different expression levels of both EGFR and RXR α .²⁶ We previously compared the expression of hypoxic markers in the same cohort(s) as the present study and only

in glottic LSCC the expression was significantly associated with LC.¹⁸ Altogether, these findings suggest that early stage supraglottic and glottic LSCC might be considered as different entities. This possible explanation is in line with the biological difference between LSCC originating from the glottis and the supraglottis. On an early embryological basis, the larynx has been subdivided into the supraglottis, developing from the buccopharyngeal sac, while the (sub)glottis develops from the tracheopulmonary sac. Moreover, exposure of the different sublocations in the larynx to carcinogens such as tobacco and alcohol cannot be considered identical and might also explain the variation in clinical outcome, genomic alterations and protein expression levels.

In many studies on the prognostic and/or predictive role of biomarkers, LSCC are included with a (often unknown) mix of glottic/supraglottic cases and might explain the observed variation of outcomes.^{8,27,28} Our data suggest that in future studies both sublocations in the larynx should be analyzed separately.

Furthermore, our cohort shows in agreement with earlier findings that lymph node positivity is a predictor for LC and OS in supraglottic LSCC.²⁹ Therefore, the significant correlation of lymph node status with LC demonstrates that (p)FADD expression is no (strong) predictor in this cohort.

CONCLUSION

In this homogeneous cohort of T1-T2 supraglottic LSCC primarily treated with radiotherapy, lymph node status was associated with local recurrence whereas the expression of pFADD was not.

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PTEN is associated with worse local control in early stage supraglottic laryngeal cancer treated with radiotherapy

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ABSTRACT

Objectives: The aim of this study was to establish the prognostic value of the epidermal growth factor receptor (EGFR) and phosphatase and tensin homolog deleted on chromosome ten (PTEN) expression on local control in patients with early stage supraglottic laryngeal squamous cell carcinoma (LSCC) treated with radiotherapy only.

Methods: Immunohistochemical staining for EGFR and PTEN was performed on pre-treatment biopsies of a selected well-defined homogeneous group of 52 patients with T1-T2 supraglottic laryngeal squamous cell carcinoma treated with radiotherapy between 1990 and 2008. Kaplan-Meier analysis, univariate and multivariate Cox Regression analysis were performed to correlate clinical data and expression levels of EGFR and PTEN with local control.

Results: Kaplan-Meier survival analysis and Cox Regression analysis showed a significant association between PTEN expression and local control (HR 3.26, 95% CI 1.14-9.33, $p=0.027$) and between lymph node status and local control (HR 3.60, 95% CI 1.26-10.31, $p=0.017$). Both were independent prognostic factors in a multivariate analysis (HR 3.28, 95% CI 1.14-9.39, $p=0.027$ and HR 3.62, 95% CI 1.26-10.37, $p=0.017$ respectively). There was no significant association between EGFR expression and local control (HR 1.32, 95% CI 1.17-10.14, $p=0.79$).

Conclusion: This study showed an association between both high PTEN expression and the presence of lymph node metastasis and deteriorated local control in early stage supraglottic LSCC treated with radiotherapy.

INTRODUCTION

Most early stage (T1-T2) laryngeal squamous cell carcinomas (LSCC) are treated with radiotherapy only. The 5-year local control rates after radiotherapy vary between 43% to 95% and 41% to 58% for glottic and supraglottic T1-T2 LSCC respectively.^{1,2} Currently, besides sublocation and stage of the primary tumor, no suitable markers are available for predicting prognosis. Cell biological markers may be helpful to select patients who may benefit from additional treatment and identify new potential targets for therapy.³

In cancer, activation of signaling pathways, including the PI3K/AKT anti-apoptotic and proliferation pathway could contribute to tumorigenesis and hence a worse prognosis.⁴⁻⁶ The PI3K/AKT pathway can be triggered by activation of the epidermal growth factor receptor (EGFR), a transmembrane glycoprotein consisting of an extracellular ligand binding domain, a transmembrane region, and an intracellular tyrosine kinase domain. Upon binding of the specific ligand, phosphorylation of the intracellular tyrosine kinase occurs, activating the PI3K/AKT pathway.^{4,7} More than 80% of the HNSCC show EGFR overexpression.⁸ It has been suggested that EGFR plays an important role in response to radiotherapy, resulting in decreased local control.⁹⁻¹² The last decade EGFR has become an important target in cancer therapy with the use of cetuximab, an anti-EGFR antibody.⁴ In HNSCC, the use of cetuximab is approved by the FDA in combination with radiotherapy for patients with locally advanced nonmetastatic HNSCC in case chemoradiation is not feasible.¹³ Unfortunately, EGFR expression does not appear to be predictive for response to EGFR inhibitors.¹⁴

Another mechanism for PI3/AKT pathway activation is the loss of PTEN (phosphatase and tensin homolog deleted on chromosome ten), a tumor suppressor gene which opposes PI3K/AKT activation.^{5,6} PTEN is the second most mutated tumor suppressor gene after p53 and mutations in PTEN are found in a variety of primary tumors including HNSCC^{7,15-18}, while germline mutations in PTEN cause Cowden syndrome, characterized by hamartomas and predisposition of breast and thyroid tumors.^{5,6} Besides the role of PTEN in tumorigenesis as an antagonist in the PI3K/AKT pathway, PTEN has also been associated with genome stability including DNA double strand break (DSB) repair by regulating the protein RAD51.^{15,19,20} Theoretically, tumors with loss of function of PTEN are less able to repair damage caused by radiotherapy. Furthermore, effect of radiotherapy is based on the high proliferation rate of tumors in relation to normal tissue. Consistent with this we previously found overexpression of PTEN was associated with increased radio resistance in a cohort of HNSCC patients postoperatively treated with radiotherapy.¹⁹ The present study was primarily designed to evaluate this association and to establish the prognostic value of EGFR and PTEN expression on local control in an independent well-documented homogeneous series of early stage supraglottic LSCC patients treated with radiotherapy only. Secondly we evaluated whether also other patient and tumor characteristics were associated with local control in this series.

MATERIAL AND METHODS

Patients

The study population of the present study was composed of a selected well-defined homogenous group of patients with stage T1-T2 histologically confirmed squamous cell carcinoma of the

supraglottic larynx treated with radiotherapy with curative intent. This study was restricted to this cohort of supraglottic tumors since previous studies showed differences in biological behavior between glottic and supraglottic tumors.²¹ The same cohort of patients was described in detail previously.^{21,22} Between 1990 and 2008, 1286 patients were diagnosed with and/or treated for a laryngeal malignancy in our institute. Of all patients demographic and clinicopathological data such as gender, age, T-status, N-status were retrospectively collected by reviewing the patient charts. The inclusion criteria for this study were (1) histologically proven squamous cell carcinoma; (2) localized in the supraglottic larynx; (3) staged cT1 and cT2; (4) with no evidence for distant metastasis (cM0); (5) were curatively treated with radiotherapy alone, and (6) received no other previous or concurrent treatment modalities. In total 247 patients with T1-T2 LSCC were retrieved in our database. Because of second primary tumors or previous chemo- and/or radiotherapy 10 patients were excluded

From this 237 patients formalin-fixed, paraffin-embedded pre-treatment biopsies taken at our institute were collected and revised by an experienced pathologist. Tissue specimens with sufficient neoplastic cells for immunohistochemical staining were available from 141 patients of which 52 were supraglottic tumors. All data from the 52 patients considered appropriate for immunohistochemistry after the exclusionary process were retrieved and collected in an anonymous database. All patients gave approval to use tissue samples for research.

Treatment

All patients were treated with primary radiotherapy as reported previously.^{21,22} In short, in stage T1 tumors, a total dose of 66 Gy, using 2 Gy fractions, five times weekly were given. Stage T2 tumors were generally treated with six fractions weekly, to a total dose of 70 Gy within 6 weeks. In the case of elective radiotherapy to the neck nodes, a total dose of 46 Gy was given to the primary planning target volume, with an additional boost of 70 Gy to the primary tumor and pathologic lymph nodes. In all patients, a planning-CT scan was made in supine position. The target volumes were delineated as described in previous reports.²³ All patients were treated with 3D-conformal radiotherapy. After completion of treatment, patients were followed at the outpatient clinic for 5 years.

Immunohistochemistry

EGFR and PTEN protein expression was respectively detected using Novocastra™ monoclonal antibody clone 113 against EGFR (Leica Microsystems Cat# NCL-L-EGFR, RRID:AB_563696) and amonoclonal antibody clone 6H2.1 against PTEN (Cascade BioScience Cat# ABM-2052, RRID:AB_2335636). Immunostaining was performed on 4 µm paraffin sections. Antigen retrieval was achieved by heating in a microwave in pre-heated Tris-HCL buffer (EGFR) and citrate buffer (PTEN). Endogenous peroxidase was blocked by incubation with 0.3% hydrogen peroxidase for 30 minutes.

Slides were stained for 1 hour with the antibody against EGFR (1:100) and PTEN (1:50). Secondary rabbit anti-mouse antibody was diluted 1:100 in 1% BSA/PBS complemented with 1% human AB serum and applied for 30 minutes at room temperature. Tertiary goat anti-rabbit antibody was diluted 1:100 and applied for 30 minutes at room temperature. Staining was visualized with 3′3′-diaminobenzidinetetrahydrochloride and counterstained with hematoxylin.

Evaluation of immunostaining

Scoring was performed by two independent teams without knowledge of clinical data. The discordant cases were reviewed and scores were reassigned on consensus of opinion. For both antibodies staining intensity was semi-quantitatively scored as negative (-), weak positive (+/-), positive (+) and strong positive (++). For PTEN the percentage of positive cells was scored as well. For statistical analysis, any positive EGFR staining above background was considered as high (+/-, + and ++).¹⁹ For PTEN, tumors were considered high in case of > 7.5% positive (+ or ++) cytoplasmic staining.¹⁹

Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics 23 for Windows. Follow-up time was calculated from the day of diagnosis until the date of the last follow-up. Local recurrence was defined as tumor recurrence at the primary tumor site within 5 years, and was calculated from the date of diagnosis until the day of local recurrence or lost to follow-up. Relations (Hazard ratio (HR) and 95% Confidence Interval (95% CI)) between recurrence and immunostaining and other clinicopathological characteristics were calculated using univariate Cox proportional hazard analysis and Kaplan-Meier analysis. For multivariate Cox regression analysis only the variables that appeared statistically significant in univariate analysis were included. P values of <0.05 were considered statistically significant.

RESULTS

Table 1. Patient and tumor characteristics of all patients (n=52).

Characteristics	No. of patients (%)
Age – years	
Median (range)	63 (33-95)
Gender	
Female	11 (21.2)
Male	41 (78.8)
cT-stadium	
T1	15 (28.8)
T2	37 (71.2)
cN-stadium	
N0	39 (75.0)
N+	13 (25.0)
EGFR expression	
Low	4 (7.7)
High	48 (92.3)
PTEN expression	
Low	39 (75.0)
High	13 (25.0)

T = tumor; N = node; EGFR = epidermal growth factor receptor; PTEN = phosphatase and tensin homolog deleted on chromosome ten.

Patient and immunohistochemical characteristics

The population showed a preference for the male sex, T2 and lymph node negative tumors (for details see Table I). Almost all tumors showed high expression of EGFR (92%). Thirteen patients (25%) had a high expression of PTEN with immunohistochemistry. In slides with high expression for PTEN the median percentage of positive neoplastic cells was 28% (range 10-85).

No relation between EGFR expression and local control

Fourteen patients (26.9%) developed a local recurrence after radiotherapy. Kaplan-Meier and Cox Regression analysis did not show a significant relation between EGFR expression and local control in our series of 52 supraglottic laryngeal carcinomas (HR 1.32, 95% CI 1.17-10.14, $p=0.79$) (Table II and Figure 1A).

Table 2. Patient characteristics, tumor characteristics, EGFR expression and PTEN expression in relation to local recurrence (n=14).

Characteristics	No. of patients with local recurrence (%)	Univariate HR (95% CI)	p-value
Age – years			
<65	8/32 (25.0)	1	
≥65	6/20 (30.0)	1.51 (0.52-4.36)	0.45
Gender			
Female	2/11 (18.2)	1	
Male	12/41 (29.3)	1.81 (0.40-8.09)	0.44
cT-stadium			
T1	3/15 (20.0)	1	
T2	11/37 (29.7)	1.63 (0.46-5.86)	0.45
cN-stadium			
N0	7/39 (17.9)	1	
N+	7/13 (53.8)	3.60 (1.26-10.31)	0.017*
EGFR			
Low	1/4 (25.0)	1.32 (1.17-10.14)	0.79
High	13/48 (27.1)	1	
PTEN			
Low	7/39 (17.9)	1	
High	7/13 (53.8)	3.26 (1.14-9.33)	0.027*

EGFR = epidermal growth factor receptor; PTEN = phosphatase and tensin homolog deleted on chromosome ten HR = Hazard ratio; CI = Confidence Interval; T = tumor; N = node . * Signifies statistically significant relation.

PTEN overexpression and lymph node status are associated with local control

In the population with high PTEN expression (n=13), 7 patients (54%) developed a local recurrence, in contrast to 18% in patients with low PTEN expression. Cox Regression revealed a significant association between high PTEN expression and local control (HR 3.26, 95% CI 1.14-9.33, $p=0.027$). (Table II and Figure 1B).

In the population with positive lymph node status (N+), 7 out of 13 patients (54%) had a local recurrence. In the population with no lymph node metastasis this percentage was 18%. Cox

Figure 1. Kaplan-Meier curves for the relation of epidermal growth factor receptor (EGFR) expression (A), phosphatase and tensin homolog deleted on chromosome ten (PTEN) expression (B) and lymph node (N) status (C) with local control.

Table 3. Results of multivariate Cox Regression analysis for local control.

Characteristics	Multivariate HR (95% CI)	P
N+	3.62 (1.26-10.37)	0.017*
High PTEN	3.28 (1.14-9.39)	0.027*

HR = Hazard Ratio; CI= Confidence Interval; N = node; PTEN = phosphatase and tensin homolog deleted on chromosome ten. * Signifies statistically significant relation.

Regression analysis revealed a significant association between N+ and local control (HR 3.60, 95% CI 1.26-10.31, $p=0.017$). (Table II and Figure 1C).

Multivariate Cox Regression showed that high PTEN expression and lymph node status were independent prognostic factors in a multivariate analysis (HR 3.28, 95% CI 1.14-9.39, $p=0.027$ and HR 3.62, 95% CI 1.26-10.37, $p=0.017$ respectively) (Table III).

DISCUSSION

Previously, we reported that overexpression of PTEN was associated with increased radio resistance in a cohort of HNSCC patients postoperatively treated with radiotherapy.¹⁹ In this study, we evaluated the prognostic value of PTEN expression on local control in an independent well-documented homogeneous series of early stage supraglottic laryngeal squamous cell carcinomas all treated curatively with radiotherapy only. In this homogeneous cohort of 52 patients a significant association between PTEN expression and local control was observed.

The last decade the PI3K/AKT signaling pathway has become an important target in cancer therapy including HNSCC with the use of cetuximab, an anti-EGFR antibody applied in advanced nonmetastatic HNSCC in combination with radiotherapy or as a single agent in patients who have had prior platinum-based therapy.¹³ EGFR status as a specific predictive marker for response to EGFR inhibitors is controversial.¹⁴ Numerous studies reported on the prognostic value of immunohistochemical EGFR expression in different tumor types.^{10,19,24} In our homogeneous cohort consisting of early stage supraglottic laryngeal squamous cell carcinomas treated with radiotherapy alone, no significant relationship between EGFR expression and local control was found. Conflicting results have been published earlier concerning EGFR overexpression and association with local control in HNSCC. For instance, Ang et al. reported worse local control and overall survival in a group of 155 advanced HNSCCs treated with radiation therapy overexpressing EGFR.¹⁰ In contrast, Eriksen et al. failed to show a significant relationship between EGFR overexpression and local control in a group of 336 HNSCC's treated with conventional or accelerated radiation therapy.²⁴ There are different reasons for these conflicting results. First, the prognostic significance of EGFR has been investigated in heterogeneous HNSCC populations treated with different treatment modalities

Second, there is no general consensus regarding the staining protocols and scoring methods of EGFR.¹⁹ The interpretation of the results in our study is difficult since there were only 4 cases with low EGFR expression. Compared to other studies on EGFR expression in HNSCC, we found a high percentage of high EGFR cases (92%) versus 53 to 88% in HNSCC described in literature.^{19, 24-28} There is only one other study concerning EGFR expression in supraglottic laryngeal carcinomas, showing high EGFR expression in 83% and 70% of T1 and T2 tumors respectively.²⁵ However, they used a different primary antibody and scoring criteria, which makes it difficult to compare. We also performed EGFR immunostaining on a well-defined series of early stage (T1-T2) glottic laryngeal squamous cell carcinomas treated with radiotherapy only and found significantly more tumors in the supraglottic group with high EGFR expression compared to the glottic group (92% vs. 53%) (Wachters et al., accepted for publication *Laryngoscope* 2020 Jan). This suggests also an association

between EGFR expression levels and different tumor locations and supports our decision to restrict our analysis to a homogeneous series of supraglottic laryngeal squamous cell carcinomas only.

Besides activation of EGFR, another mechanism for PI3/AKT pathway activation is the loss of PTEN expression. Previously, PTEN expression has been linked to therapeutic outcome in HNSCC.^{19, 29-31} In this study we showed a significant relation between high PTEN expression and worse local control. Low PTEN expression by immunostaining was observed in 75% of patients similar as we observed earlier in HNSCC.¹⁹ Lower percentages of 33-56% using the same antibody were reported as well^{32,33}, in which supraglottic LSCC appeared to have less often loss of expression than glottic LSCC.³³ This is possibly explained by the more often raised disease of supraglottic LSCC.³³ These percentages are corresponding to mutations and loss of heterozygosity of the PTEN gene found in 23-33% and 10-40% of HNSCC respectively.¹⁶⁻¹⁸ However, a study in non-small-cell lung cancer showed that neither methylation nor loss of heterozygosity was a significant predictor of PTEN protein expression with immunohistochemistry.³⁴ Among several types of commercially available PTEN monoclonal antibodies, PTEN monoclonal antibody clone 6.H2.1 was the only one associated with PTEN molecular alterations.³⁵

The results of this study confirmed the previously found relation between high expression of PTEN and worse local control in a series of squamous cell carcinomas of the larynx, hypopharynx, oropharynx and oral cavity treated with primary surgery and postoperative radiotherapy.¹⁹ The explanation for this association was that PTEN functions in double strand break (DSB) repair.²⁰ Cells have evolved several protective responses to counteract the harmful effects of DNA-damage including Homologous Recombination (HR) and Non-Homologous End-Joining (NHEJ) for DSB. HR ensures accurate DSB-repair mediated through the so called RAD52 group of proteins which includes RAD51. RAD51 is regulated by PTEN.^{15,19,20} We speculate that tumors with low function of PTEN are less able to repair damage caused by radiotherapy. Other studies on PTEN expression in relation with prognosis in HNSCC have linked, on the contrary, low PTEN to worse prognosis of disease after both surgery and radiotherapy.²⁹⁻³¹ An explanation for this observation is the counteracting function of PTEN on the PI3K/AKT anti-apoptotic and proliferation pathway that could contribute to tumorigenesis and worse prognosis.^{5,6} Positive lymph node status was associated with the development of local recurrence. This was an unexpected outcome because regional metastasis is not a known risk factor for developing local recurrence and suggest the higher aggressiveness of these tumors in general. It was not in relation with tumor size because there was no difference in outcome between T1 and T2 tumors. The relation of regional metastasis and deteriorated local control should be evaluated in larger series of early stage supraglottic LSCC.

This study was weakened by its small series of only 52 patients. On the other hand, the great strength of this study was the composition of a well-defined homogeneous cohort consisting of supraglottic laryngeal squamous cell carcinomas treated with radiotherapy alone. The relevance to distinguish between supraglottic and glottic LSCC evaluating (prognostic) immunohistochemical expression of antibodies has become clear from previous studies.^{21,22,33,36} Although we performed EGFR immunostaining on a series of early stage glottic LSCC as well, we did not performed PTEN immunostaining on this glottic LSCC series. It was quite difficult to obtain a useful immunohistochemical visualization of PTEN on the supraglottic LSCC biopsies.

Therefore we decided not to sacrifice the mostly small biopsies from the glottic LSCC patients. One of the reasons for radio resistance in HNSCC is accelerated repopulation of tumor cells after exposure to ionizing radiation.^{9,24,25,37} One of the mechanisms contributing to this repopulation is activation of the EGFR/PI3K/AKT pathway. Binding of cetuximab to the EGF receptor leads to internalization and degradation of the antibody-receptor complex, downregulating EGFR expression.^{9,38} In SCC cell lines Bonner showed that the combination of cetuximab and radiation resulted in decreased cellular proliferation.³⁹ Bonner et al. showed that among patients with stage III-IV HNSCC, the addition of cetuximab to radiotherapy resulted in a significant improvement of locoregional control and survival as compared to radiotherapy alone, without enhancing radiation-induced toxicity.^{40,41} Based on the results of this study, cetuximab is now increasingly used in combination with definitive radiotherapy in locally advanced disease in particular for patients in which concurrent chemoradiation is considered not feasible. So far, no studies have been published which compared concomitant cetuximab and radiotherapy in combination with EGFR levels in early stage laryngeal carcinoma.

This study showed a potential role in diagnostic and prognostic terms for PTEN immunohistochemical evaluation in supraglottic LSCC, in which patients with supraglottic LSCC with a high PTEN immunohistochemical expression might benefit from altered radiotherapy schedules or additional PTEN inhibiting drugs. Because of the antagonizing behavior of PTEN on the PI3/AKT pathway most of the strategies to target PTEN are focused on PTEN-deficient cancers utilizing inhibitors of kinases that lie at the level of PI3K/AKT pathway.⁴² Furthermore, due to the activation of the EGFR/PI3/AKT pathway, PTEN inactivation could theoretically lead to resistance to EGFR inhibitors. Frattini et al. showed in a series of colorectal cancer patients that loss of PTEN expression by immunohistochemistry distinguished responder from nonresponder patients to cetuximab.⁴³ Studies in prostate cancer cells showed a comparable relation and PTEN reintroduction restored the cell response to cetuximab.⁴⁴ This supports the suggestion that supraglottic LSCC patients with high PTEN expression might benefit from adding cetuximab to radiotherapy.

CONCLUSION

This study confirmed the earlier found association between high PTEN expression and local control in HNSCC. The worse local control found in cases with high PTEN expression might suggest the importance of PI3K-AKT independent functions of PTEN such as DNA-damage repair in radioresponse. PTEN status could have an additive value in determining the prognosis of early stage supraglottic laryngeal carcinomas, next to the already used clinical factors as lymph node status. These patients might benefit from an alternated radiation schedule, drugs interfering with DNA-damage repair or adding cetuximab.

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Distinct biomarker profiles and clinical characteristics in T1-T2 glottic and supraglottic carcinomas

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ABSTRACT

Background: In early-stage laryngeal squamous cell carcinoma (LSCC) radiotherapy with curative intent is a major treatment modality. TNM classification is used to define patients eligible for radiotherapy. Studies in early-stage glottic LSCC identified several predictive biomarkers associated with local control. However, we recently reported that this predictive value could not be confirmed in supraglottic LSCC.

Objective: To examine whether clinical behavior and protein expression patterns of these biomarkers differ between glottic and supraglottic LSCC.

Study Design: Retrospective cohort study.

Methods: Tumor tissue sections of 196 glottic and 80 supraglottic T1-T2 LSCC treated primarily with RT were assessed immunohistochemically for expression of pAKT, Ki-67 and β -Catenin. Expression data of HIF-1 α , CA-IX, OPN, FADD, pFADD, Cyclin D1, Cortactin and EGFR in the same cohort of glottic and supraglottic LSCC, were retrieved from previously reported data. The relationship between glottic and supraglottic sublocalization and clinicopathological, follow-up and immunohistochemical staining characteristics were evaluated using logistic regression analyses and Cox regression analyses.

Results: Glottic LSCC were correlated with male gender ($p=0.001$), hoarseness as a primary symptom ($p<0.001$), T1 tumor stage ($p<0.001$), negative lymph node status ($p<0.001$) and an older age at presentation ($p=0.004$). Supraglottic LSCC patients developed more post-treatment distant metastasis when adjusted for gender, age and T-status.

While supraglottic LSCC was associated with higher expression of HIF-1 α ($p=0.001$), Cortactin ($p<0.001$), EGFR ($p<0.001$) and Ki-67 ($p=0.027$), glottic LSCC demonstrated higher expression of CA-IX ($p=0.005$) and Cyclin D1 ($p=0.001$).

Conclusion: Differences in clinicopathological and immunohistochemical staining characteristics suggest that T1-T2 glottic and supraglottic LSCC should be considered as different entities.

INTRODUCTION

Head and neck cancer encompasses a broad spectrum of malignancies, and is responsible for 550,000 new cases and 380,000 deaths worldwide annually.¹ Histologically, the vast majority (approximately 90%) concerns mucosal squamous cell carcinomas. Head and neck squamous cell carcinoma (HNSCC) is a heterogeneous group of malignancies, involving multiple subsites. About 21% of all HNSCC are located in the larynx.² Squamous cell carcinoma of the larynx (LSCC) originates from supraglottic, glottic and subglottic sites in 69%, 28% and 1% respectively (2% overlapping/unknown).³

In early-stage LSCC radiotherapy is a major treatment modality. However, local control (LC) rates vary between 40 and 100% in early-stage LSCC.⁴ Radiotherapy failure frequently results in highly morbid salvage surgery. A major wound complication rate of 60% and a pharyngocutaneous fistula rate of 30% has been described.⁵

TNM staging, apparently, is of insufficient predictive value towards radio response. In search for new predictive markers in LSCC, immunohistochemical expression of tumor specific proteins has been investigated frequently. We^{4, 6-8} and others⁹⁻¹¹ studied proteins such as those involved in cell cycle regulation, tumor hypoxia and cell adhesion. However, the predictive value of these markers varied considerably. For instance, in separate homogeneous cohorts of 91 glottic and 60 supraglottic T1-T2 LSCC treated with primarily radiotherapy, hypoxia markers HIF-1 α and CA-IX were predictive for LC in glottic LSCC⁷, but not in supraglottic LSCC.⁴ More recently we found that pFADD overexpression was associated with a significantly improved LC rate in glottic LSCC⁸, whereas in our supraglottic cohort it was not.⁶ One explanation for this discrepancy is that these LSCC sublocalizations represent other entities, each with its own clinical and biological behavior. As a consequence, the protein expression patterns might differ.

In this study, we will compare the frequency of high/low expression of hypoxia markers HIF-1 α , CA-IX and OPN, the 11q13 related biomarkers FADD, pFADD, Cyclin D1 and Cortactin, and EGFR in a large cohort of pretreatment biopsies of 196 glottic and 80 supraglottic T1-T2 LSCC. The expression of most of these markers was studied previously in association with clinical outcome upon radiotherapy in this cohort of glottic and supraglottic early-stage LSCC. The data on frequency of expression of these markers were retrieved from previously published studies.^{4, 6-8, 12} In addition, we selected 3 other biomarkers from the literature because of their typical profile in LSCC (pAKT, Ki-67 and β -Catenin)¹³ and performed expression analysis by immunohistochemistry using the same cohort of pretreatment biopsies of 196 glottic and 80 supraglottic T1-T2 LSCC.

MATERIAL AND METHODS

Patient and biomarker selection

A database was constructed with 1513 patients treated in the University Medical Center Groningen for (pre-)malignant laryngeal disease between 1990 and 2011. The following selection criteria were used, as reported previously^{4, 6}: histologically confirmed squamous cell carcinoma restricted to the glottic and supraglottic region; staged cT1 or cT2; curatively treated with primarily radiotherapy

with no other previous treatment; sufficient formaldehyde-fixed paraffin-embedded (FFPE) pretreatment tumor material, resulting in a cohort of 650 patients. Five patients were excluded because of a malignancy of the head and neck region in their medical history, nine patients were excluded because of the existence of a double tumor in the head and neck region and 11 patients were excluded because of chemotherapy or other systemic treatment of malignancies before or during treatment for their LSCC.

From 276 patients sufficient FFPE tumor tissue was available, covering 196 glottic and 80 supraglottic LSCC patients (Table 1). The collection of patient data and tissue samples was approved by the Medical Ethics Committee of our hospital.

The cohort of patients treated between 1990-2008 was described in detail in previously reported studies on the expression of HIF-1 α , CA-IX, OPN, FADD, pFADD, Cyclin D1, Cortactin and EGFR in relation to response to radiotherapy.^{4,6-8,12} For the expression analysis of pAKT, Ki-67 and β -Catenin, the cohort was expanded with early-stage LSCC patients treated between 2009 and 2011.

Immunohistochemistry

First, hematoxylin and eosin staining was performed after 4 μ m serial sections were cut, to evaluate whether sufficient tumor material was available for immunohistochemistry.

Antigen retrieval was achieved by heating in a microwave in preheated citrate buffer (pH 6.0) for 15 minutes at 100 °C, by incubation overnight in Tris-HCl (pH 9.0) at 80 °C and by heating in a microwave in preheated Tris/HCL buffer for 15 minutes at 100 °C respectively. Next, endogenous peroxidases were blocked for 30 minutes at room temperature (RT) with 0.3% H₂O₂. The slides were incubated at RT for 1 hour with antibodies against pAKT (1:50, clone 736E11, Cell Signalling Massachusetts, USA), Ki-67 (1:350, Clone MIB-1, Dako, Glostrup, Denmark) and β -Catenin (1:1000, clone 14/B, BD Biosciences, New Jersey, USA). Subsequently, for pAKT immunostaining Envision treatment was applied (Dako, Glostrup, Denmark) for 30 minutes at RT. For Ki-67 and β -Catenin stainings secondary antibodies were applied for 30 minutes at RT (1:100, RAMPO, Dako, Glostrup, Denmark), followed by tertiary antibodies, as well for 30 minutes at RT (1:100, GARPO, Dako, Glostrup, Denmark). The peroxidase reaction was performed by applying 3,3'-diaminobenzide tetrachloride for 10 minutes, followed by counterstaining with hematoxylin, dehydration and mounting.

Evaluation of immunohistochemical staining

All slides were scored by 2 observers separately, blinded for follow-up data. In general, in this study protein expression is classified as high when the percentage of positive tumor cells is higher than the pre-defined cut-off.

For evaluation of cytoplasmic pAKT immunostaining, expression levels (high or low) were defined using a cut-off of 10% of tumor cells with positive expression, as reported previously in HNSCC.¹³⁻¹⁶ The evaluation of Ki-67 expression has been performed using different cut-offs, varying from 10-60%.¹⁷⁻²¹ Therefore, Ki-67 expression was evaluated using the median staining percentage (49%). Expression of β -Catenin was scored on both membranous as well as cytoplasmic staining. For membranous staining a cut-off percentage of 10% was used, as reported previously¹³. In case of β -Catenin cytoplasmic staining any staining above background staining was considered positive

as reported earlier.^{11, 22} Examples of pAKT, Ki-67, β -Catenin and EGFR immunostainings are shown in Fig. 1a-d.

Expression data of HIF-1 α , CA-IX, OPN, FADD, pFADD, Cyclin D1, Cortactin and EGFR in our two cohorts of glottic and supraglottic LSCC were retrieved from our previous studies.^{4, 6-8, 12, 23} The used antibodies, the immunohistochemical staining conditions and the scoring criteria of the immunostainings of all used biomarkers are summarized in supplementary data, Table S1.

Statistical analysis

All statistical analyses were performed using the SPSS 23.0 software (Armonk, NY, USA: IBM Corp.). For analyzing relations between clinicopathological characteristics and immunohistochemical staining characteristics on the one side and glottic/supraglottic LSCC sublocalization on the other side, uni- and multivariate logistic regression analyses were used. Follow-up data were analyzed using uni- and bivariate Cox regression analyses. P-values of <0.05 were considered to be statistically significant.

RESULTS

Differences in clinicopathological and follow-up data in T1-T2 glottic and supraglottic LSCC

We compared common clinicopathological and follow-up data observed in relation with tumor sublocalization in our cohort of pretreatment biopsies of 196 glottic and 80 supraglottic LSCC patients (Table 1). This analysis revealed that duration of complaints was not correlated significantly with LSCC sublocalization ($p=0.414$). However, male gender ($p=0.001$), hoarseness as a primary symptom ($p<0.001$), T1 tumor stage ($p<0.001$), negative lymph node status ($p<0.001$) and an older age of tumor presentation ($p=0.004$) were statistically significantly correlated with glottic sublocalization. On multivariate logistic analysis these findings remained statistically significant (Table 1).

Both LSCC sublocalizations demonstrated a similar number of patients with locoregional recurrences (supplementary data, Table S2). Adjusted bivariate Cox regression analyses revealed no significant difference in the case of locoregional recurrence (Table 2). Patients with supraglottic LSCC developed more distant metastasis after initial diagnosis/treatment ($p=0.009$), losing significance when adjusted for primary symptom ($p=0.203$) and N-status ($p=0.114$). More supraglottic LSCC patients died, which was only significant when adjusted for age ($p=0.015$). Significance of death of disease (DOD) was present only when additionally corrected for gender ($p=0.035$).

Comparison of expression profiles of biomarkers associated with clinical outcome between glottic and supraglottic LSCC

In order to validate differences between glottic and supraglottic T1-T2 LSCC, we compared the frequency of expression of these 11 selected biomarkers (pAKT, Ki-67, β -Catenin, HIF-1 α , CA-IX, OPN, FADD, pFADD, Cyclin D1, Cortactin and EGFR), reported as potential prognostic or predictive markers towards clinical outcome in LSCC (summarized in Table 3). The percentage of tumor cells with high expression in both the glottic and supraglottic sublocalization was similar for OPN, FADD, pFADD, pAKT and β -Catenin. The percentage of supraglottic LSCC with high expression of HIF-1 α , Cortactin,

Table 1. Description of pretreatment clinical characteristics, stratified by supraglottic (n=80) and glottic LSCC (n=196).

Characteristic	Value	Supraglottic LSCC	Glottic LSCC	Univariate log. regression OR (95%CI)*	Multivariate log. regression OR (95%CI)*
Gender	Male Female	59 (73.8) 21 (26.3)	177 (90.3) 19 (9.7)	1 3.32 (1.67-6.59)**	1 3.53 (1.37-9.13)**
Age (years)	<64 >64 Median (Range)	53 (66.3) 27 (33.7) 62.0 (33-96)	92 (46.9) 104 (53.1) 65.0 (35-89)	1 0.45 (0.26-0.78)**	1 0.34 (0.15-0.77)**
Primary symptom	Hoarse voice Other	39 (48.8) 41 (51.3)	191 (97.4) 2 (0.5)	1 100.40 (23.30-432.52)***	1 128.40 (26.65-618.56)***
Duration of complaint (weeks)	<22 >22 Median (Range)	42 (53.8) 36 (46.2) 18.0 (0-520)	94 (48.7) 99 (51.3) 24.0 (0-520)	1 0.81 (0.48-1.38)	NA
T-status	T1 T2	22 (27.5) 58 (72.5)	100 (51.0) 96 (49.0)	1 2.75 (1.56-4.83)***	1 2.98 (1.33-6.68)**
N-status	N0 N+	61 (76.3) 19 (23.8)	192 (98.0) 3 (1.5)	1 19.93 (5.70-69.67)***	1 8.54 (1.67-43.70)**
N-status	N1 N2 N3 Nx	9 (11.3) 9 (11.3) 1 (1.3) 0 (0.0)	3 (1.5) 0 (0.0) 0 (0.0) 1 (0.5)	NA	NA

Abbreviations: LSCC = laryngeal squamous cell carcinoma; CI = confidence interval; OR = odds ratio; T = tumor; N=node; NA = not applicable * Presented are N(%), unless specified otherwise. Performed were logistic regression analyses where the presence of supraglottic LSCC was considered as the outcome. **p<0.05. *** p<0.001.

Table 2. The impact of LSCC sublocalization (supraglottic versus glottis) on outcome (locoregional recurrence, distant metastasis, overall death and death of disease).*

Correction for	Sublocalization	Locoregional recurrence HR (95%CI)	Distant metastasis HR (95%CI)	Death HR (95%CI)	Death of disease HR (95%CI)
Non-adjusted estimate	Glottic LSCC	1	1	1	1
	Supraglottic LSCC	1.14 (0.68-1.90)	4.95 (1.49-16.50)**	1.33 (0.92-1.93)	0.54 (0.25-1.19)
Gender	Glottic LSCC	1	1	1	1
	Supraglottic LSCC	1.29 (0.77-2.17)	5.19 (1.53-17.65)**	1.41 (0.96-2.06)	0.40 (0.17-0.94)**
Age	Glottic LSCC	1	1	1	1
	Supraglottic LSCC	1.17 (0.69-1.97)	5.40 (1.56-18.64)**	1.61 (1.10-2.35)**	0.56 (0.25-1.26)
Primary symptom	Glottic LSCC	1	1	1	1
	Supraglottic LSCC	1.25 (0.67-2.34)	2.78 (0.58-13.44)	1.31 (0.83-2.06)	0.50 (0.17-1.47)
T-status	Glottic LSCC	1	1	1	1
	Supraglottic LSCC	1.02 (0.60-1.73)	5.14 (1.51-17.51)**	1.32 (0.91-1.91)	0.50 (0.23-1.11)
N-status	Glottic LSCC	1	1	1	1
	Supraglottic LSCC	0.91 (0.51-1.60)	2.93 (0.77-11.09)	1.23 (0.83-1.81)	0.70 (0.23-2.13)

Abbreviations: LSCC = laryngeal squamous cell carcinoma; HR = hazard ratio; CI = confidence interval

*Cox regression analysis adjusted for gender, age, primary symptom, T-status and N-status. ** $p < 0.05$.

Table 3. Results of immunohistochemistry, logistic regression analysis.

	Staining characteristics	Glottic LSCC	Supraglottic LSCC	Logistic regression OR (95%CI)*	Previously reported
HIF-1α	Low n (%)	45 (49.5)	13 (21.7)	1	Schrijvers 2008 161, Wachters 2013
	High n (%)	46 (50.5)	47 (78.3)	3.54 (1.69-7.41)**	
	Median (range)	1.0 (0-60)	6.0 (0-50)		
CA-IX	Low n (%)	54 (59.3)	49 (81.7)	1	Schrijvers 2008 161, Wachters 2013
	High n (%)	37 (40.7)	11 (18.3)	0.33 (0.15-0.71)**	
	Median (range)	10.0 (0-90)	1.0 (0-40)		
OPN	Low n (%)	69 (76.7)	40 (66.7)	1	Wachters 2013
	High n (%)	21 (23.3)	20 (33.3)	1.64 (0.80-3.40)	
	Median (range)	0.0 (0-50)	0.0 (0-80)		
FADD	Low n (%)	71 (77.2)	40 (66.7)	1	Schrijvers 2012 1220, Wachters 2017
	High n (%)	21 (22.8)	20 (33.3)	1.69 (0.82-3.49)	
	Median (range)	2.0 (0-4)	2.0 (0-4)		
pFADD	Low n (%)	30 (32.6)	17 (28.3)	1	Schrijvers 2012 1220, Wachters 2017
	High n (%)	62 (67.4)	43 (71.7)	1.22 (0.60-2.49)	
	Median (range)	80 (20-100)	80.0 (5-100)		
Cyclin D1	Low n (%)	43 (49.4)	46 (78.0)	1	Glottic: Schrijvers 2012
	High n (%)	44 (50.6)	13 (22.0)	0.28 (0.13-0.58)**	
	Median (range)	10.0 (0-85)	35.0 (0-95)		
Cortactin	Low n (%)	42 (51.9)	12 (20.3)	1	Glottic: Schrijvers 2012
	High n (%)	39 (48.1)	47 (79.7)	4.22 (1.95-9.10)***	
	Median (range)	20.0 (0-100)	65.0 (0-100)		
pAKT	Low n (%)	48 (34.3)	14 (22.6)	1	NA
	High n (%)	92 (65.7)	48 (77.4)	1.79 (0.90-3.57)	
	Median (range)	50 (0-100)	70 (0-100)		
EGFR	Low n (%)	41 (47.1)	3 (5.9)	1	Supraglottic: Bruine de Bruin 2019
	High n (%)	46 (52.9)	48 (94.1)	14.26 (4.13-49.28)***	
	Median (range)	2 (1-4)	2 (1-4)		
Ki-67	Low n (%)	76 (56.7)	25 (39.7)	1	NA
	High n (%)	58 (43.3)	38 (60.3)	1.99 (1.08-3.66)**	
	Median (range)	48.0 (15-86)	51 (4-89)		
β-Catenin (membr.)	Low n (%)	6 (4.4)	4(6.3)	1	NA
	High n (%)	131 (95.6)	60 (93.8)	0.69 (0.19-2.52)	
	Median (range)	75.0 (0-100)	76.5 (0-100)		
β-Catenin (cytopl.)	Low n (%)	129 (94.2)	63 (98.4)	1	NA
	High n (%)	8 (5.8)	1 (1.6)	0.26 (0.03-2.09)	
	Median (range)	0 (0-1)	0 (0-1)		

Abbreviations: LSCC = laryngeal squamous cell carcinoma; CI = confidence interval; OR = odds ratio; NA not applicable

* Logistic regression analysis for the presence of supraglottic LSCC. ** $p < 0.05$. *** $p < 0.001$.

EGFR and Ki-67 was significantly higher, compared to glottic LSCC ($p=0.001$, $p<0.001$, $p<0.001$, $p=0.027$ respectively). On the other hand, expression of CA-IX and Cyclin D1 was significantly increased in glottic LSCC compared to supraglottic LSCC ($p=0.005$, $p=0.001$ respectively).

Figure 1. Positive staining for pAKT (A), EGFR (B), Ki-67 (C) and β -Catenin (D) in laryngeal squamous cell carcinoma. Original magnification, 200x..

DISCUSSION

Despite many studies on the prognostic and/or predictive value of immunohistochemical biomarkers in HNSCC cohorts, conflicting and divergent results keep these biomarkers from being incorporated in daily strategies. This might be explained by population diversity in TNM stages, immunohistochemical techniques, staining evaluation methodologies and anatomical tumor sublocalizations²⁴⁻²⁶.

Previously, we studied the association between local control and expression of several markers in separate homogeneous cohorts of T1-T2 glottic and supraglottic LSCC, all treated with radiotherapy only. This association with some markers observed in the glottic cohort^{7,8}, could not be confirmed in the supraglottic cohort.^{4,6} Recently, we found that pATM expression was associated with local control in supraglottic but not in glottic LSCC.²⁷ These observations suggested that within these tumor sublocalizations, clinical and biological behavior and hence protein expression patterns might differ.

In this study we analyzed both the clinicopathological characteristics and the differences in frequency of high expression of these biomarkers in two homogeneous, well-defined cohorts of 196 glottic and 80 supraglottic LSCC, all diagnosed and treated at the same institute. To circumvent that differences are the result of technical issues, all tissue samples were collected from the same cohort, immunohistochemistry of all markers was performed in the same lab using similar conditions and all immunostainings were evaluated using the same scoring criteria as previously defined (supplementary data, Table S1).^{4, 7, 8, 12, 13, 23}

From an epidemiological perspective, male gender was significantly more dominant in the glottic than in the supraglottic LSCC patients. This finding is in concordance with other studies^{28, 29}, whereas in several studies gender was not related with tumor sublocalization.^{30, 31} Geographical differences in the use of alcohol between both sexes are known confounders.³² Moreover, compared to the (sub)glottis, the supraglottis is exposed to a relatively higher degree to ingested agents and to a lesser degree to inhaled agents³³, suggesting that etiological factors, like the use of alcohol and tobacco, might have distinguishable epidemiological effects. Because of the notoriously unreliable and frequently lacking data on the use of tobacco and alcohol in our cohorts, these characteristics were not analyzed in our study.

In the literature differences between the age at which early-stage glottic and supraglottic LSCC was diagnosed, have not been described. In our study both the median age and mean age (65.19 and 62.04 resp., data not shown) were 3 years higher in glottic LSCC patients. Significantly more patients in our supraglottic cohort deceased during our follow-up period, which is in agreement with the literature³, although disease-related death rates were similar (supplementary data Table S2). However, in both glottic and supraglottic LSCC only a minority died of disease related causes.

From a clinical perspective, differences between glottic and supraglottic LSCC can be pointed out as well. Relatively small mucous membrane disruptions on a glottic level produce vibratory disturbances of the involved vocal fold during speech and hence hoarseness in the patient, whereas comparable mucous membrane disturbances on a supraglottic level can remain unnoticed or misinterpreted by the patient for a longer period of time.³⁴ This is in good agreement with the

observation that hoarseness was a significantly more frequently experienced primary symptom in our glottic LSCC cohort. Additionally it can explain the significantly higher T-stage at presentation in our supraglottic cohort.

Moreover, already on an early embryological basis, the glottic and supraglottic larynx have distinguishable origins. The glottic and subglottic larynx are derived from the sixth branchial arch and the supraglottis from the third and fourth branchial arch.^{35, 36} The supraglottic compartment ultimately develops a rich lymphatic network and, with relatively few anatomical barriers, forming a rather easy entrance for locoregional tumor spread. Tumor spread in the glottic compartment however is hampered by elastic layers and a more limited network of lymphatics.³⁷ The observed higher lymph node status at diagnosis in supraglottic T1-T2 LSCC as compared to glottic LSCC is in agreement with this organic perspective and with the literature as well.³⁸

On a molecular level, the literature demonstrates some clues that protein expression profiles between glottic and supraglottic LSCC might differ. One study, using a relatively small series of 35 glottic and 25 supraglottic T1-T4 N0-N+ LSCC, revealed a significantly higher EGFR and RXRa expression in glottic LSCC, whereas expression of NF- κ B and Cox2 were similar in both supraglottic and glottic LSCC.³⁹ Another study showed a decreased expression of β -Catenin in supraglottic LSCC, but no difference regarding ILK, pAKT, E-Cadherin, Vimentin, AR and Er-b).¹³ These findings suggested the existence of distinguishable protein expression profiles in glottic and supraglottic LSCC. On the other hand, α -Catenin, CD44, hyaluronan, p53 and Bcl-2 was not correlated with tumor sublocalization within 136 glottic and 62 supraglottic Tis-T2 LSCC cohort.³⁷

In the present paper, we selected 11 biomarkers, frequently reported in LSCC/HNSCC and often reported to be associated with clinical outcome.^{4, 6-8, 23, 40} Differential expression between glottic and supraglottic LSCC was observed for 6 markers (HIF-1 α , CA-IX, Cyclin D1, Cortactin, EGFR and Ki-67) (Table 3). Tumor hypoxia is an extensively studied process in HNSCC and has been shown to induce genetic instability, tumor cell aggression and treatment failure.^{41, 42} Predominantly, endogenous hypoxia markers HIF-1 α and its down-stream upregulated intracellular pH regulating CA-IX have been under investigation concerning their relation with survival and locoregional control.⁴¹ We observed that the number of cases with expression of both HIF-1 α and CA-IX was significantly different between glottic and supraglottic LSCC (Table 3). In the literature, expression of CA-IX was not investigated in glottic and supraglottic LSCC separately, whereas 2 reports on expression of HIF-1 α did not find a difference between these sublocalizations.^{43, 44} This discordance with our data might be explained by the fact that these 2 studies are composed of T1-T4 LSCC with a relatively low number of T1-T2 cases (n=41, n=63 resp.). Both studies used a cut-off value of 10%. Our predetermined cut-off value was 0.5%, as in our earlier studies, based on its relation with treatment response. Yet, when a cut-off value of 10% was applied, results remained similar (HR 2.88 CI 1.24-6.69 p=0.014, data not shown). The distribution of the expression of hypoxia markers is a good explanation that the hypoxia markers HIF-1 α and CA-IX were predictive for local control in glottic LSCC⁷, but not in supraglottic LSCC.⁴ Because tumor hypoxia can be counteracted by breathing carbogen (98% O₂ and 2% CO₂) and nicotinamide (a vasoactive drug) during radiotherapy⁴⁵ to improve treatment response, also the localization of squamous cell carcinomas in the larynx should be taken into account in treatment-decisions-making.

In HNSCC, amplification of chromosome region 11q13.3 is a frequently observed event.^{46,47} It has been associated with poor prognosis.⁴⁸ Genes for FADD, Cyclin D1 and Cortactin are located within this region, resulting in frequent overexpression in almost all HNSCC with this amplification.⁴⁹ Although FADD and pFADD expression did not show significant differences in our glottic and supraglottic cohort, Cyclin D1 and Cortactin did. Comparative studies on FADD, pFADD and Cortactin expression in glottic and supraglottic LSCC separately have not been published before, other than our previous publications.^{6,8} Several studies on Cyclin D1 expression do report on glottic and supraglottic LSCC separately.⁵⁰⁻⁵⁴ None of these studies revealed expression differences between tumor sublocalization, but the number of T1-T2 LSCC cases in most of these study populations was rather small (n=46, n=75, n=38, n=21, n=8, respectively). Evaluation methods and cut-off percentages in the literature referred to above, differed (3-point scale, immunoreactive score, 5% cut-off, 5-point scale, 10% cut-off resp.). As in previous studies in LSCC, we utilized a cut-off of 32.5%.⁸

The AKT-pathway is a complex signal transduction pathway that promotes cell cycle regulation in response to extracellular signals. Both pAKT and EGFR are key proteins in this pathway and the corresponding protein expression has been demonstrated to be of clinical significance in HNSCC.^{55,56} For EGFR staining we utilized the methods of Pattje et al.²³ Complete circular membranous staining was considered high expression and no or incomplete circular membranous staining was considered low expression. Four reports analyzed the expression of EGFR in the LSCC sublocalizations separately, all using different staining evaluation methodologies.^{39,53,57,58} Only one publication described a significantly increased frequency of tumors with high expression of EGFR in glottic LSCC.³⁹ In our cohorts, a significantly increased frequency of cases with high expression of EGFR was found in supraglottic LSCC. The population of Kourelis et al. was however too small to draw firm conclusions, containing only 18 glottic and 12 supraglottic early-stage LSCC. Regarding the expression of pAKT, only Goulioumis et al. evaluated glottic and supraglottic LSCC separately and described no significantly differing expression pattern,¹³ which is in agreement with our results. Cetuximab, a monoclonal antibody targeting the extracellular domain of EGFR, has been in use in clinical praxis in advanced stage HNSCC.⁵⁹ As high expression of EGFR was found particularly in supraglottic LSCC, anti-EGFR treatment might be more effective in LSCC originating from the supraglottic larynx.

Ki-67 is a good marker to quantify the degree of proliferative activity in neoplastic cells, since nuclear Ki-67 is present in all phases of the cell cycle, but not in the G0 phase (resting cells).⁶⁰ The nuclear expression of Ki-67 was significantly higher in our supraglottic cohort compared to our glottic cohort, confirming the findings of Tamas et al.⁶¹ There are publications without significant association between Ki-67 expression and LSCC sublocalization as well.^{62,63} Study populations existed of T1-T4 or unknown stage LSCC and differing evaluation methods were used, complicating a firm comparison.

In LSCC, loss of expression of the membrane protein β -Catenin, has been associated with tumor dedifferentiation and a decrease in disease-specific survival.^{22,64} In a group of 46 glottic and 12 early-stage supraglottic LSCC loss of membranous β -Catenin expression was associated with supraglottic sublocalization.¹³ Moreover, a combined cytoplasmic and membranous β -Catenin expression was associated with supraglottic sublocalization in a population of 32 LSCC of unknown TNM stage.¹¹ In our LSCC cohorts β -Catenin did not demonstrate a sublocalizational difference, following the results of Greco et al.²²

CONCLUSION

Our data on protein expression patterns and clinicopathological features in a well-defined cohort of 276 early-stage LSCC suggest that supraglottic and glottic LSCC should be considered as different entities. This is in good agreement with differences observed in tumor response upon radiotherapy and clinical outcome. Our findings imply that results of reported studies that have included both glottic and supraglottic LSCC should be interpreted with caution. In future studies, we strongly recommend to evaluate supraglottic and glottic LSCC separately. Ultimately, these plausible biological differences between glottic and supraglottic LSCC may lead to more differential treatment schedules in (subpopulations of) glottic and supraglottic LSCC.

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Summary
General Discussion
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SUMMARY

The commonly-used TNM classification system is not fully capable of predicting response to radiotherapy in HNSCC. Recurrent or residual HNSCC after radiotherapy often necessitates salvage surgery. Salvage surgery can have devastating consequences for patients in terms of surgical complications and hospital admittance.¹⁻³ Many studies have been reported on evaluating the prognostic and predictive value of immunohistochemical tumor markers towards clinical outcome in HNSCC. However, variation within patient populations considering HNSCC sublocalization, TNM stage (tumor expansion, lymph node status, distant metastasis), treatment modality, technique of immunohistochemistry and its scoring evaluation, seem to blur results and hamper the implementation of new tumor markers in daily clinical practice.^{4,5}

In our research group, in the last decade, the predictive and prognostic value of several immunohistochemical markers in T1-T2 glottic LSCC patients curatively treated with only radiotherapy was investigated (chapter 1).^{6,7} These tumor markers were selected because of their (possible) role in the clinical outcome upon treatment in LSCC and other HNSCC and these markers are involved in cancer-related pathways such as tumor hypoxia (HIF1a, CA-IX, OPN), cell cycle regulation (FADD, pFADD, Cyclin D1) and the EGFR-PI3K-AKT-pathway (EGFR, pAKT). In our previous research, mainly LSCC from the glottis were studied. Because of the possibly distinct biology of glottic and supraglottic LSCC, the first goal was to evaluate whether the markers tested in glottic LSCC that showed an association with local control upon radiotherapy, also demonstrated a similar predictive and prognostic value in a homogeneous well-documented cohort of T1-T2 supraglottic LSCC patients, also treated with primary radiotherapy.

In chapter 2 associations between expression of endogenous hypoxia markers HIF-1a and CA-IX and clinical outcome, using immunohistochemistry on pretreatment tumor biopsies was investigated. The hypothesis was that the predictive and prognostic value of HIF-1a and CA-IX, found in the glottic LSCC cohort was comparable in the supraglottic cohort. However, neither HIF-1a nor CA-IX were of prognostic significance towards local control or overall survival in T1-T2 supraglottic LSCC. More recently, two studies directly investigated HIF-1a and CA-IX in early stage LSCC. The first study described that high levels of HIF-1a and CA-IX were significantly related with local recurrence in 13 supraglottic and 29 glottic T1-T2 LSCC.⁸ Another study demonstrated in a large cohort of 286 T1-T2 glottic LSCC that HIF-1a and CA-IX were not of predictive/prognostic value.⁹ In this latter study, the majority of patients is, however, of the T1a stage, a stage only scarcely included in our cohort, since internationally T1a glottic LSCC patients are preferably treated by endoscopic surgery. Additionally, in a third study, the mRNA levels of HIF-1a were associated with tumor grade, mode of invasion, tumor recurrence and overall survival in a non-specified group of LSCC.¹⁰ The exact working mechanism and clinical significance of HIF-1a remains to be elucidated.

F-FAZA-PET/CT scanning, using a hypoxic tracer, was introduced in 2007 for the detection of hypoxia in HNSCC tumors.^{11, 12} In Chapter 3, F-FAZA-PET/CT scanning was performed in 11 advanced-stage or recurrent LSCC patients in a pilot study to evaluate the association of F-FAZA-PET/CT scans with expression of exogenous and endogenous hypoxic markers simultaneously.

Using these F-FAZA-PET/CT scans inter- and intratumoral heterogeneity of tumor hypoxia was observed. After total laryngectomy, F-FAZA-PET/CT scanning based hypoxic regions were selected for immunohistochemistry towards exogenous (pimonidazole) and endogenous (HIF1 α , CA-IX and GLUT-1) markers of hypoxia. Hypoxia was detected with pimonidazole, HIF1 α , CA-IX and GLUT-1 expression in some selected hypoxic tumor regions. However, no clear overlap was observed between (18)F-FAZA uptake and immunohistochemical hypoxia markers. Hence, the accuracy of (18)F-FAZA-PET/CT scanning as a marker for hypoxia could not be proved.

Since our publication in 2015, such a study, investigating the spacial resolution of (18)F-FAZA-PET scanning has not been published. Only few clinical studies involved (18)F-FAZA-PET/CT scanning, with promising results though. In 2016, Kinoshita et al. demonstrated in localized non-small cell lung cancer patients that (18)F-FAZA uptake was associated with tumor aggression (e.g. vascular invasion, pleural invasion, larger tumor diameter, advanced stage) and clinical outcome (poorer overall and progression free survival).¹³ Another study demonstrated (18)F-FAZA-PET/CT scanning to be predictive for radiotherapy response in a esophageal adenocarcinoma xenograft model.¹⁴ Although associations with endogenous hypoxia markers and other exogenous hypoxia markers could not be demonstrated in our study, the true predictive/prognostic value of (18)F-FAZA-PET/CT scanning in LSCC needs to be investigated in larger populations.

The chromosome region 11q13 is amplified in HNSCC in approximately 35% of cases and the amplified region harbors several genes including the FADD gene.¹⁵ Previously, we reported that the expression of the phosphorylated isoform of the FADD protein (pFADD), was associated with a significantly improved local control in T1-T2 glottic LSCC.⁷ In chapter 4, the analysis of pFADD expression in our cohort of supraglottic LSCC revealed no association with local recurrence. Since the publication of our study, the predictive value of (p)FADD has not been studied in LSCC or HNSCC in other studies.

In stage 1-4 oropharyngeal SCC a similar association between pFADD expression and local control was found¹⁶, as observed in glottic LSCC (Schrijvers 2012 1220).⁷ In addition, Pattje et al demonstrated in vitro an association between pFADD-expression, G2M cell cycle arrest and radiosensitivity.¹⁶ At this moment we do not have an explanation for the lack of an association in supraglottic LSCC. Expression of the non-phosphorylated FADD isoform has been reported to be associated with a higher incidence of lymph node metastasis at presentation and with shorter distant metastasis free survival when lymph node metastases are present in HNSCC¹⁷, but FADD expression did not affect the cell cycle arrest and radiosensitivity in vitro.¹⁶ One explanation for the difference in predictive/prognostic value of (p)FADD could be that the relative expression levels of pFADD and FADD might influence clinical outcome. More research is needed to further clarify the exact role of pFADD towards cell cycle control and hence radio sensitivity.

Many studies in HNSCC have reported a major role of the activation of the EGFR pathway in relation to clinical outcome and treatment response. For example, Pattje et al showed in a series of 167 T1-T4 oral and oropharyngeal squamous cell carcinoma, all surgically treated with postoperative radiotherapy that of the essential components of the EGFR-PI3K-AKT pathway, not EGFR but expression of PTEN was associated with locoregional control.¹⁸ In chapter 5, the association between expression of EGFR and PTEN, and local control in our cohort of supraglottic LSCC patients was

tested. Expression of EGFR was not associated with clinical outcome, but high expression of PTEN was associated with a deteriorated local control. This is in very good agreement with our previous findings.¹⁸

Glottic and supraglottic LSCC demonstrate distinct epidemiological and clinical behavior (chapter 1). Several tumor markers reported to be associated with local control (LC) in glottic LSCC, were not predictive in our cohort of supraglottic LSCC. The question rises whether glottic and supraglottic LSCC represent distinct entities with their own biological behavior on a molecular level as an explanation for the differences in the predictive/prognostic value of tumor markers. In order to investigate whether glottic and supraglottic LSCC should be considered as separate entities, in Chapter 6, the expression profiles of many relevant tumor markers were evaluated in the same LSCC cohorts with glottic and supraglottic LSCC cases from 2008-2011 added. For this purpose, previously reported expression data of HIF-1a, CA-IX, OPN, FADD, pFADD, Cyclin D1, Cortactin and EGFR were re-evaluated. Based on a literature search, 3 additional markers that were not tested before in our group (pAKT, Ki-67 and β -Catenin) were included. Expression was determined by immunohistochemistry on the same pretreatment biopsies of our glottic and supraglottic LSCC cohorts. Glottic sublocalization was correlated with several clinical parameters such as male gender, hoarseness as a primary symptom, T1 tumor stage, negative lymph node status and an older age of tumor presentation. Patients with supraglottic LSCC developed more distant metastasis after initial diagnosis/treatment when adjusted for gender, age and T-status. In supraglottic LSCC, the percentage of HIF-1a, Cortactin, EGFR and Ki-67 was significantly higher, compared to glottic LSCC. On the other hand, expression of CA-IX and Cyclin D1 was significantly increased in glottic LSCC. Differences in clinicopathological and immunohistochemical staining characteristics between T1-T2 glottic and supraglottic LSCC suggest that supraglottic and glottic LSCC should be considered as different entities, each with its own distinct clinical and biological behavior. This could also be an explanation for the observed differences in the association with clinical outcome for various biomarkers in HNSCC and LSCC in the literature. Our findings also imply that results of reported studies that have included both glottic and supraglottic LSCC, both early and advanced stage, should be interpreted with caution. Therefore we strongly recommend to study supraglottic and glottic LSCC separately in future LSCC marker research.

GENERAL DISCUSSION

Embryological, anatomical and clinical considerations

The larynx starts developing during the fourth week of gestation. From a developing laryngeal orifice in the foregut a respiratory longitudinal diverticulum develops, eventually forming a tubular structure, cranially surrounded by branchial arches. The supraglottis is derived from the third and fourth branchial arches (buccopharyngeal primordium). The glottis and subglottis are derived from the 6th branchial arch (tracheobronchial primordium) and are formed by the union of lateral walls that develop on each side of the tracheobronchial primordium (Figure 1).^{19, 20}

Although this embryologically distinct basis of laryngeal development is frequently described and referred to in medical literature and handbooks²⁰⁻²³, it has been questioned as well. De Bakker et al. claim that thyroid and cricoid cartilages develop from mesenchymal condensations and not from specific branchial arches.²⁴

Figure 1. Formation of the respiratory diverticulum from the 4th week of gestation onward (A, B, C), originating from the laryngeal orifice at the level of the 4th branchial arch (D). Figure adapted from profelis.org.

The internal lining of the larynx originates from endoderm. However, ultimately the mucosal lining differs in the supraglottic, glottic and subglottic region. As in the case of the subglottic and tracheal region, the epithelium of the supraglottic region is predominantly of the pseudostratified columnar type, except at the edges of the aryepiglottic folds and the lateral borders of the epiglottis, which are lined by stratified squamous epithelium. Especially the supraglottic region has an abundance of mucous glands. The glottic region, however, is lined with only stratified squamous epithelium, devoid of mucous glands. In the case of the free edge of the true vocal cords, this stratified squamous epithelium covers a 3-layered lamina propria and an underlying thyroarytenoid muscle and vocal ligament, giving it its vibratory properties enabling vocal activity, but all together supposedly forming a barrier for tumor spread as well.²⁵ This ingenious laryngeal vocal physiology is easily disturbed, already by only small superficial anomalies, leading to a hoarse and/or breathy voice.^{22, 26} Most glottic LSCC are detected therefore in an early stage (approximately 85%), whereas in the case of supraglottic LSCC only a minority (approximately 30%).²⁷

The supraglottic compartment has a rich lymphatic network and because the supraglottis is embryologically formed without a midline union, its lymphatics drain bilaterally. On the other hand, the glottic region is embryologically formed by paired structures that fuse in the midline and as a result, the only sparse lymphatic network drains unilaterally. Therefore, typically, compared to supraglottic LSCC, glottic LSCC nodal involvement at the moment of diagnosis occurs more often only unilaterally and less frequently.^{20, 28}

Epidemiological considerations

In the Netherlands, approximately 700 patients are diagnosed with LSCC annually, with a gender distribution demonstrating a striking male predominance in glottic LSCC (90%) and in a lesser degree in supraglottic (74%) and subglottic LSCC (82%).^{27, 28} Although risk factor behavior could explain this male predominance, a role for sex hormones in the development of LSCC has been postulated, but remains to be elucidated.²⁹

Worldwide, the distribution of supraglottic and glottic LSCC differs geographically, whereas subglottic LSCC, rather steadily comprises 1-3% of all laryngeal cancers.³⁰ As in most northern European countries, in The Netherlands, the glottis is classically affected more often than the supraglottis (ratio: 2:1), in contrast with certain Latin American and Mediterranean countries, in which supraglottic localization is found more frequently.^{27, 31} These geographical differences are thought to be caused by differences in risk factor exposure. Although excessive consumption of alcohol and smoking tobacco (active and passive) are the main risk factors for developing LSCC^{32, 33}, especially excessive alcohol consumption is more closely related with the development of supraglottic LSCC compared to (sub)glottic LSCC.³⁴

Considerations towards treatment and prognosis

As outlined in chapter 1, early stage LSCC can be treated with either endoscopic surgery or radiotherapy. In the last two decades, endoscopic surgery in early stage LSCC has been gaining popularity. Transorally, with or without the use of a laser, LSCC can be resected. It is a minimally invasive technique compared to open surgery, preserving laryngeal function. As in the University Medical Center Groningen, in the literature T1a glottic LSCC is generally thought to be treated preferably by primary endoscopic surgery.³⁵ In case of failure of (potentially multiple attempts of) endoscopic surgery, radiotherapy remains a main treatment modality.

Reported local control rates in early stage LSCC after radiotherapy vary widely for both sublocalizations. In glottic and supraglottic LSCC local control rates after radiotherapy, vary between 61-94% and 74-100% respectively. Reported 5-year disease specific survival rates vary between 86-98% and 76-100% respectively.³⁶

In the literature, there are clues that even carcinomas in subregions of the glottic and supraglottic larynx demonstrate differences on clinical outcome after radiotherapy. Eskiizmir et al. reported a systematic review illustrating that T1-T2 glottic LSCC tumors with anterior commissure involvement had a significantly higher risk of radiotherapy failure.³⁷ Other studies described deteriorated clinical outcome for T1 glottic LSCC with involvement of the posterior commissure³⁸ and the superior aspect of the vocal cord.³⁹ On the supraglottic level, subregional differences have been investigated in only

one study. In 111 T1-T4 supraglottic LSCC survival rates were better for patients with carcinomas of the epiglottis or ventricular bands compared to those with carcinoma of the aryepiglottic fold or ventricle.⁴⁰

Pathological and molecular considerations

Histologically, LSCC is generally divided into 3 grades (well, moderately and poorly differentiated) based on the formation of keratin and the presence of intercellular bridges, cellular pleomorphism and mitotic activity. This histological grading system has been ascribed prognostic value, but grading is subjective and sampling error may have been of influence.⁴¹ Independent stage adjusted differences between glottic and supraglottic LSCC have not been described in the literature.

The microvessel density, reflecting the amount of angiogenesis within the tumor, has generally been correlated with the ability of tumors to grow and metastasize.⁴² However, no significant microvessel density differences have been demonstrated in glottic and supraglottic LSCC.⁴²⁻⁴⁵

In the last 15 to 20 years more and more evidence has accumulated demonstrating that a number of types of human papillomavirus are etiologically involved in a subset of HNSCC, especially oropharyngeal squamous cell carcinoma. In LSCC however, high-risk HPV(hrHPV)-positivity has been demonstrated to be only 1.5% vs. 18.5% in oropharyngeal carcinomas, suggesting that hrHPV does not have a large impact on the development, clinical behavior and/or clinical outcome of LSCC.⁴⁶ However, the review of Nowinska et al. suggests the incidence of hrHPV-infected LSCC might be significantly higher depending on the procedure applied for sample collection and the viral DNA detection method. Although hrHPV infected oropharyngeal squamous cell carcinomas have a less severe course of disease and are characterized by longer patient survival, in the literature there are no clues suggesting such an association in LSCC.^{47, 48} Significant differential grades of hrHPV-infection within the LSCC sublocalizations have not been described in the literature.

There is only a very limited number of publications focusing directly on molecular differences between glottic and supraglottic LSCC. A study in 2002 reported that expression of α -Catenin, CD44, hyaluronan, p53 and Bcl-2 was not correlated with tumor sublocalization within their cohort of 136 glottic and 62 supraglottic Tis-T2 LSCC.²⁵ On the other hand, Kourelis et al. conducted a study in 35 glottic and 25 supraglottic T1-T4 N0-N+ LSCC concerning a variety of potential biomarkers (EGFR, NF- κ B, RXRa and COX-2), demonstrating that glottic localization was significantly associated with intense EGFR and RXRa expression ($p=0.011$, $p=0.001$ respectively).²³ And finally, Goulioumis et al. investigated several other potential biomarkers (ILK, pAKT, β -Catenin, E-Cadherin, Vimentin, AR, ER- β) and found that supraglottic tumor localization was statistically significantly correlated with a decreased expression of β -Catenin ($p=0.025$).²²

More indirectly, several studies collaterally compared glottic and supraglottic LSCC marker expression patterns in search for potential prognostic and/or predictive value. Table 1 summarizes the literature, covering sublocalizational differences and similarities of tumor

Table 1. Summary of glottic/supraglottic tumor marker expression comparisons in the literature..

	Publication, no association with LSCC sublocalization	Population	Publication, positive association with LSCC sublocalization	Population
HIF-1a	Da-Wei Li 2013 75 Xiao-Hong Wu 2013 261	84 T1-T4 LSCC 46 T1-T4 LSCC	None	NA
CA-IX	None	NA	None	NA
OPN	Yong Li 2012 1429 Chen 2015 7116	54 T1-T4 LSCC 105 T1-T4 LSCC	None	NA
FADD	None	NA	None	NA
pFADD	None	NA	None	NA
Cyclin D1	Rasamni 2012 923 Wildeman 2009 316 Kamal Morshed 2007 760 Yi-Fei Liu 2013 1137 Rehab Allah Ahmed 2014 625 Jovanovic 2014 944	84 T1-T4 LSCC 59 T1-T3 LSCC 130 T1-T4 LSCC 116 T1-T4 LSCC 75 T1-T4 LSCC 48 T1-T4 LSCC	Zhi-Jie Fu 2008 (borderline positive assoc. with gl. LSCC)	58 T1-T4 LSCC
Cortactin	None	NA	None	NA
pAKT	Gouliouris 2008 511 Gouliouris 2010 1573 Nijkamp 2013 349	97 T1-T4 LSCC 97 T1-T4 LSCC 78 T2-T4 LSCC	None	NA
EGFR	Nijkamp 2013 3202 Bussu 2012 1724 Wildeman 2009 316	272 T2-T4 LSCC 67 T1-T4 LSCC 59 T1-T3 LSCC	Kourelis 2008 265 (positive assoc. with gl. LSCC)	61 T1T4 LSCC
Ki-67	Fuat Adikalin 2004 376 Bai 2016 2641 Zhang 2016 433	63 T1-T4 LSCC 50 T1-T4 LSCC 165 T1-T4 LSCC	Tamas 2011 721 (positive assoc. with supragl. LSCC)	79 LSCC (stage unknown)
β-catenin	Greco e148 2016 Nardi 2018 30199 Zhu 2018 3472 Mezi 2017 1806	82 T1-T4 LSCC 45 T1-T4 LSCC 76 T1-T4 LSCC 50 T1-T4 LSCC	Gouliouris 2010 1573 (positive assoc. with gl. LSCC) Galera Ruiz 2012 1185 (positive assoc. with gl. LSCC)	97 T1-T4 LSCC 16 LSCC (stage unknown)

marker expression in glottic and supraglottic LSCC. Most positive associations between marker expression and tumor sublocalization, are counterbalanced with one or more studies demonstrating no significant association with sublocalization. Populations vary from 16 to 272 cases, mostly covering T1-T4 LSCC cases. None of the studies exclusively covers early stage LSCC, making our study population evaluated in chapter 6 a unique homogeneous cohort. Interestingly, we showed that differences between both clinicopathological and immunohistochemical staining characteristics in glottic and supraglottic LSCC are in very good agreement with other studies and, consequently, should be considered clinically and biologically as distinct entities.

FUTURE PERSPECTIVES

Distinct laryngeal entities

The findings in this thesis demonstrated that T1-T2 glottic and supraglottic LSCC should be regarded as different entities and that in future studies on predictive and prognostic markers glottic and supraglottic LSCC should be evaluated separately. To unravel the different tumor-specific pathways needed for predictive/prognostic purposes and ultimately exploring new treatment modalities, it would be interesting to study differences between these two tumor types using whole-genome-sequence analysis, expression profiling and proteomics. Such approaches were, for instance, used to investigate the complexities around hrHPV-positive oropharyngeal squamous cell carcinoma.⁴⁹

General considerations towards marker research

For improving local control and ultimately survival rates in cancer patients in general, treatment modalities need to be improved and/or the TNM classification system needs supportive data, capable of predicting therapy response. Especially with LSCC, a TNM classification system with extended tumor marker evaluation could enable selection of subsets of patients with poor outcome for alternative treatment. On the other hand, markers with predictive or prognostic value might help to gain insight in mechanisms of cancer development or the basis of treatment failure. In line with this assumption, alternative or customized treatment modalities can be proposed based on protein expression, potentially interfering with tumor associated protein pathways or sensitizing tumor cells for systemic treatment or radiotherapy. Regarding existing treatment strategies in LSCC, examples for improvement might be based on targeting known affected pathways in HNSCC such as hypoxia, cell cycle regulation and growth factor regulation and the very promising novel possibilities in immunotherapy.

Targeting tumor hypoxia might improve clinical outcome in LSCC

Tumor hypoxia is a common feature in HNSCC and is associated with radio-resistance, chemo-resistance and deteriorated clinical outcome.⁵⁰⁻⁵² Several therapies to counteract tumor hypoxia induced treatment resistance have been proposed.⁵¹

Inhibition of HIF-1a has been reported in the literature, but to date there is no FDA-approved inhibitor available.^{53, 54} Bhattarai et al reported several potential HIF-1a inhibitors, still in their early stages of development, most of which inhibitory mechanisms are partially undefined and/or indirect.⁵³ Indirect HIF-1a inhibition functions by blocking the HIF-1a transcription/translation or by promoting HIF-1a protein degradation.^{53, 54} For future research direct and hence selective inhibition of HIF-1a by preventing transactivation, DNA binding and transcriptional activity of HIF-1a, is mandatory for unraveling the exact potential of HIF-1a inhibition.

Hypoxia-enhanced cytotoxins and radiosensitizers are interesting potential targeted treatment additives.⁵¹ For example, tirapazamine is a hypoxic cell cytotoxin and is reduced to a reactive radical under hypoxic conditions, damaging DNA by single- and double-strand breaks.⁵⁵ Hypoxic cell radiosensitizers, such as nimorazole, act as oxygen mimetics, inducing free radical formation and hence sensitizing hypoxic cells to the cytotoxic effects of ionizing radiation.⁵¹

In the case of tirapazamine, few trials have been performed in advanced HNSCC. There is a phase I and a phase II trial demonstrating improved clinical outcome for addition of Tirapazamine to chemoradiotherapy^{56, 57}, but there is a phase III trial demonstrating a lack of additional benefit towards clinical outcome.⁵⁸ However, in these studies, HNSCC sublocalizational stratification was not performed and the presence of tumor hypoxia was not a patient selection criterium for the 2 treatment arms. Le et al demonstrated, in advanced HNSCC, associations between clinical outcome after chemoradiotherapy with or without Tirapazamine and hypoxia-related tumor markers IL-8 and HGF.⁵⁹ A comparable clinical trial, using HIF-1a or CA-IX in homogeneous glottic LSCC cohorts would be an interesting topic for future research.

Nimorazole significantly improved the effect of conventional radiotherapy treatment schedules in early and advanced stage supraglottic and pharyngeal tumors without major side-effects.⁶⁰ Metwally et al demonstrated similar findings in a clinical trial.⁶¹ However, the study was terminated due to accrual problems. In these clinical trials, hypoxia marker evaluation (using intrinsic and/or extrinsic markers of hypoxia) was not a patient selection criterium. Whether selection of patients with hypoxic tumors results in a further improvement of clinical outcome remains to be elucidated. There is an on-going phase III trial in locally advanced HNSCC of the oropharynx, hypopharynx and larynx, in which hypoxia will be taken into account.⁶² A hypoxia gene signature will be validated within their HNSCC cohort. It has not been described whether tumor sublocalization, as a differentiating factor, will be taken into consideration.

In our glottic cohort, HIF-1a and CA-IX were of predictive and prognostic value, suggesting that hypoxia in early stage glottic LSCC is clinically relevant. In our supraglottic cohort, HIF-1a and CA-IX expression had no predictive/prognostic value for radiotherapy. For future research it might be interesting to study the predictive/prognostic value of HIF-1a and CA-IX in the supraglottic subregions (epiglottis, false vocal cords, ventricles, laryngeal aspect of aryepiglottic folds and arytenoids) separately, since subregional differences in HPV-expression, lymph node metastasis rate and clinical outcome, have been described.⁴⁰

The EGFR-PI3K-AKT pathway is clinically relevant

Cetuximab, a monoclonal antibody targeting the extracellular domain of EGFR, has been the first and only approved therapy in advanced stage HNSCC, interfering with the EGFR-PI3K-AKT pathway.⁶³ Binding leads to internalization and eventually degradation of the antibody-receptor complex.⁶⁴ Cetuximab is already in clinical use and the NCCN Guidelines describe cetuximab as an optional primary systemic therapy with concurrent radiotherapy in the case of advanced stage LSCC.⁶⁵ Whether there would be a role for cetuximab and concurrent radiotherapy in early stage LSCC could be an interesting subject for future studies. An important hurdle to introduce EGFR targeted therapy in early stage HNSCC is that today there is no predictive marker to identify those patients that benefit from cetuximab and to prevent treating those that would not benefit from cetuximab treatment, but only suffer from the often severe adverse effects.^{63, 64} Remarkably, despite many efforts, expression or activation of the targeted protein, EGFR itself, is not predictive. More detailed tumor marker analysis of the complete EGFR signaling pathway might provide new predictive markers. Also identifying EGFR-targeted resistant mechanisms might improve selection of patients that would not benefit from such treatment.

High expression of PTEN was significantly associated with a deteriorated local control. This is in very good agreement with our previous findings¹⁸ and illustrates the possible role of PTEN in responses to radiotherapy in HNSCC. The proliferative and antiapoptotic activities associated with PTEN downregulation on the one side^{66, 67} and the genome stabilizing activities (by RAD51) associated with PTEN upregulation and hence potentially loss of radiosensitivity, demonstrate the Januz-face of PTEN.^{18, 68, 69} The fact that in a unique homogeneous cohort of T1-T2 supraglottic LSCC treated with primarily radiotherapy only, PTEN was of predictive value towards local control (Chapter 5), raises the question whether PTEN would be of comparable predictive value in T1-T2 glottic LSCC. Since in other types of tumors, including colorectal cancer and prostate cancer, PTEN expression was associated with improved cetuximab response^{70,71}, future research could be aimed at the use of cetuximab in tumors overexpressing PTEN.

Other predictive/prognostic markers and therapeutical targets

In general, cellular protein pathways are extremely complex processes with countless interactions. It is very unlikely that there will be one "holy grail" marker predicting therapy response in LSCC. Probably, a substantial number of marker evaluations, other than the markers mentioned above, will be needed to obtain sufficient predictive value to be of additional clinical significance. Consequently, targeted therapies are probably only of use in subgroups of patients.

However, there are promising pathways and cellular interactions under investigation that have been gaining interest the last few years, such as Immune-Checkpoint Inhibition. In many malignancies, treatment with immune-checkpoint inhibitors such as pembrolizumab, nivolumab and atezolizumab, revealed very promising durable responses in subsets of the patients and acceptable toxicities.⁷²⁻⁷⁴

A dozen studies on immune-checkpoint inhibitors have been performed in mainly recurrent or metastatic HNSCC, demonstrating improvement of clinical outcome with long-lasting results and a favorable toxicity profile, but with a low response rate.^{75, 76} The reported response rate for pembrolizumab, nivolumab and atezolizumab varies between 13.3% and 35.6%.⁷⁵ Therefore, a selection of patients that respond well to immune-checkpoint inhibition would be highly beneficial. These very promising first data warrant to investigate these new immune-checkpoint inhibitors in LSCC.

Only two studies investigated the expression of the PD-L1 protein and related markers in LSCC (not treated with Immune-checkpoint Inhibition). Yu et al demonstrated in 69 T1-T4 LSCC, treated with a variety of treatment modalities, PD-L1 to be associated with lymph node metastasis and tumor differentiation. However, it did not influence tumor grading and 3-year overall survival.⁷⁷ Moreover, Vassilakopoulou et al. demonstrated in 260 T1-T4 LSCC, as well treated with by a variety of treatment modalities, PD-L1 expression to be associated with a superior disease-free and overall survival.⁷⁸

In this perspective, it would be interesting to evaluate PD-1 and PD-L1 expression in tumor tissue in homogeneously treated cohorts of glottic and supraglottic LSCC patients, early stage and late stage, in order to explore a potential role for antibodies targeting PD-1 and PD-L1, such as pembrolizumab, nivolumab and atezolizumab.

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Nederlandse samenvatting
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NEDERLANDSE SAMENVATTING

Over het algemeen worden kwaadaardige tumoren geclassificeerd op basis van tumorgrootte en uitzaaiingen naar lymfklieren of andere organen (het TNM-classificatiesysteem). Deze TNM-classificatie is een belangrijke klinische methode voor het voorspellen van het ziekteverloop en daarmee ook voor de keuze van het type behandeling. Helaas is dit classificatiesysteem niet volledig in staat terugkerende tumorgroei na behandeling te voorspellen als het gaat om plaveiselcelcarcinomen in het hoofd-halsgebied (HNSCC).

Bestraling is een veel toegepaste behandeling bij HNSCC. Terugkerende tumorgroei na bestraling kan vaak alleen nog maar behandeld worden met salvage chirurgie, een vaak zeer uitgebreide operatie, die verstreckende gevolgen kan hebben in termen van ernstige problemen bij wondheling (fisteling, infectie, noodzaak tot aanvullende chirurgische procedures), lange ziekenhuisopnames en zelfs slechtere overleving.

Er is veel onderzoek gedaan naar de voorspellende waarde van de mate van expressie (voórkomen) van kanker-gerelateerde eiwitten in weefselbiopten van tumoren, ook wel tumormarkers genoemd, met het oog op lokale controle (is en blijft de tumor na bestraling weg?) en overleving in patiënten met HNSCC. Echter, variaties in de samenstelling van onderzoekspopulaties met betrekking tot de verschillende tumorlokalisaties in het hoofd-halsgebied en verschillen in TNM-stadiëring, type behandeling en technieken om de expressie van tumormarkers te meten, lijken niet tot éénduidige resultaten te kunnen leiden. Dit lijkt het ontdekken van nieuwe tumormarkers en het uiteindelijke gebruik ervan in de dagelijkse praktijk in de weg te staan.

Om die reden is in onze onderzoeksgroep in de afgelopen 10 jaar de voorspellende waarde van vele verschillende tumormarkers onderzocht in een betrekkelijk zuivere onderzoekspopulatie, namelijk patiënten met vroeg-stadium kanker van alleen de ware stembanden (glottis), die behandeld zijn met bestraling (hoofdstuk 1). De in glottische tumoren onderzochte tumormarkers werden geselecteerd vanwege hun mogelijke voorspellende waarde met betrekking tot lokale controle en overleving. De geselecteerde tumormarkers zijn betrokken bij verschillende aan kankercellen-gerelateerde processen zoals tumorhypoxie (HIF1a, CA-IX, OPN, GLUT-1, pimonidazole), stoornissen in de regulatie van de celcyclus (FADD, pFADD, Cyclin D1) en de EGFR-PI3K-AKT-signaaltransductie (EGFR, pAKT, PTEN).

De glottis is een deelgebied van het strottenhoofd. Het deelgebied vlak boven de glottis is de supraglottis (strottenklep, valse stembanden) en onder de glottis bevindt zich de subglottis (zegelringkraakbeen). Bekend is dat tumoren van de supraglottis, glottis en subglottis klinische verschillen laten zien op het gebied van het klachtenpatroon, de risicofactoren, de neiging tot uitzaaien en het voórkomen ervan in de samenleving.

Vanwege dit klinische onderscheid en daarmee ook de mogelijke biologische verschillen tussen deze supraglottische, glottische en subglottische tumoren, was het doel van dit onderzoek om te evalueren of de eerder in glottische tumoren onderzochte tumormarkers dezelfde voorspellende waarde lieten zien in supraglottische tumoren. Daartoe werd voor het onderzoek, beschreven in dit proefschrift, een homogeen en goed gedocumenteerde groep patiënten met een vroeg-stadium

supraglottische tumor, behandeld met alleen bestraling, geselecteerd uit een database van alle patiënten met HNSCC, die behandeld werden in het UMC Groningen tussen 1990 en 2011 en jarenlang gevolgd zijn, waardoor we weten wat het effect van de behandeling geweest is.

In hoofdstuk 2 wordt in deze groep patiënten met een supraglottische tumor, de relatie tussen de expressie van hypoxiemarkers HIF-1a, CA-IX en lokale controle onderzocht, gebruikmakend van tumorweefselbiopten van vóór de bestraling. In tegenstelling tot de voorspellende waarde van HIF-1a en CA-IX, die gevonden werd in glottische tumoren, was noch HIF-1a noch CA-IX van voorspellende waarde in de supraglottische tumoren.

Naast het meten van hypoxie door eiwitanalyses in tumorweefselbiopten, kan tumorhypoxie ook onderzocht worden met F-FAZA-PET/CT-scans, waarbij gebruik gemaakt wordt van een radioactief gelabelde hypoxiemarker (F-FAZA) die met een infuus bij de patiënt vóór het maken van de scan toegediend wordt. Deze techniek werd geïntroduceerd in 2007 voor het evalueren van hypoxie in HNSCC. Hoofdstuk 3 beschrijft een pilot study, waarin een F-FAZA-PET/CT-scan verricht werd in 11 patiënten met een relatief grote HNSCC tumor. Na deze scan werd het strottenhoofd inclusief de tumor operatief verwijderd. Op het uitgenomen preparaat werden vervolgens eiwitanalyses verricht, gericht op hypoxie. Op de F-FAZA-PET/CT-scans werden, wat betreft de mate van tumorhypoxie, niet alleen verschillen gezien tussen de verschillende tumoren, maar ook binnen de tumoren zelf. Na het operatief verwijderen van het strottenhoofd werd het preparaat (het strottenhoofd) in een laboratorium systematisch in grote plakken opgesneden. Op basis van de beelden van de F-FAZA-PET/CT-scan werden verschillende wel- en niet-hypoxische gebieden geselecteerd binnen de tumor en in het preparaat opgezocht, gebiopteerd en onderworpen aan eiwitanalyses met betrekking tot hypoxiemarkers (HIF-1a, CA-IX, GLUT-1 en pimonidazole). Aankleuring van deze 4 bekende hypoxiemarkers werd waargenomen in enkele van de geselecteerde tumorgebieden. Echter, van een duidelijk overlap van F-FAZA-opname en de hypoxiemarkers was geen sprake. De nauwkeurigheid van de F-FAZA-PET/CT-scan als een hypoxiemarker kon dus niet worden bevestigd. In het DNA van ongeveer 35% van hoofd-halstumoren komt amplificatie van de chromosoomregio 11q13 voor, wat het één van de meest-voorkomende genetische afwijkingen maakt in HNSCC. Een gevolg van deze genetische amplificatie (letterlijk: vermeerderen van genen) is dat de genen die in deze chromosoomregio liggen vaker tot expressie worden gebracht, wat vaak leidt tot (overmatige) vorming van specifieke eiwitten. In tumoren met een hoge expressie kunnen deze genen meer invloed uitoefenen op verschillende tumorbiologische processen binnen de tumorcel, zoals bijvoorbeeld celcyclusregulatie. Eén van de genen in deze chromosoomregio is het FADD-gen. Het corresponderende FADD-eiwit kan door een ander eiwit worden gefosforyleerd waardoor pFADD ontstaat. Eerder werd al door onze onderzoeksgroep gerapporteerd dat de aanwezigheid van pFADD in verband gebracht kon worden met lokale controle in patiënten met een vroeg-stadium glottische tumor, behandeld met alleen bestraling. In hoofdstuk 4 wordt beschreven dat in supraglottische vroeg-stadium tumoren, behandeld met alleen bestraling, de aanwezigheid van pFADD géén relatie heeft met lokale controle. Een verklaring voor het verschil in voorspellende waarde van pFADD zou kunnen zijn dat niet de absolute, maar de relatieve mate van aanwezigheid van pFADD en FADD gerelateerd zijn aan lokale controle. Meer onderzoek is nodig om de exacte rol van (p)FADD binnen de celcyclusregulatie en daarmee de bestralingsgevoeligheid van tumorcellen

op te helderen. Net als het onderzoek beschreven in hoofdstuk 2 aangaande de relatie tussen hypoxiemarkers en lokale controle, maakt deze studie zichtbaar dat supraglottische en glottische tumoren beschouwd moeten worden als verschillende tumortypen.

Cellulaire processen worden voor een groot deel aangestuurd door ketens van met elkaar reagerende eiwitten. Eén van die ketens behelst de EGFR-PI3K-AKT-signaaltransductie. Veel onderzoeken in HNSCC hebben de EGFR-PI3K-AKT-signaaltransductie gerelateerd aan klinische uitkomstparameters, zoals lokale controle en overleving. Bij dit cellulaire proces zijn niet alleen EGFR, PI3K en AKT betrokken zoals de naam doet vermoeden, maar vinden interacties plaats met tientallen andere eiwitten, zoals bijvoorbeeld PIP2 en PTEN. Onze onderzoeksgroep liet eerder zien dat niet zozeer de mate van aanwezigheid van EGFR, maar van PTEN gerelateerd was aan lokale en regionale controle, in een patiëntengroep van 167 mond- en keelholtetumoren, allen operatief behandeld gevolgd door bestraling. In hoofdstuk 5 wordt de relatie tussen de aanwezigheid van PTEN en EGFR en lokale controle onderzocht in opnieuw patiënten met een vroeg-stadium supraglottische tumor. De aanwezigheid van EGFR kon niet in verband worden gebracht met lokale controle of overleving, maar de expressie van PTEN was wél geassocieerd met een slechtere lokale controle. Dit ondersteunt onze eerdere bevindingen in mond- en keelholtetumoren.

Glottische en supraglottische tumoren vertonen klinische verschillen (klachtenpatroon, neiging tot uitzaaien etc.), epidemiologische verschillen (risicofactoren en het vóórkomen in de samenleving) (hoofdstuk 1). Zoals beschreven in hoofdstuk 2 en 4 verschilt de voorspellende waarde van tumormarkers tussen glottische en supraglottische tumoren. De vraag rijst of glottische en supraglottische tumoren verschillende ziekten zijn, elk met zijn eigen biologisch gedrag op moleculair niveau als verklaring voor de gevonden verschillen in voorspellende waarde van tumormarkers. Om te onderzoeken of glottische en supraglottische tumoren beschouwd moeten worden als biologisch verschillend, wordt in hoofdstuk 6 de mate van aanwezigheid van de verschillende relevante tumormarkers geëvalueerd in dezelfde glottische en supraglottische tumoren. Hiertoe werden de eerder gerapporteerde gegevens van HIF-1a, CA-IX, OPN, FADD, pFADD, Cyclin D1, Cortactin en EGFR van deze glottische en supraglottische tumoren opnieuw geanalyseerd. Op basis van de beschikbare literatuur werden aanvullend een drietal tumormarkers (pAKT, Ki-67 en β -Catenin) getest in onze glottische en supraglottische tumoren. De mate van aanwezigheid van deze tumormarkers werd ook hier geanalyseerd op dezelfde tumorbipten van vóór de bestraling. Tevens werden klinische gegevens (geslacht, leeftijd, type lichamelijke klachten en aanwezigheid van uitzaaiingen naar lymfklieren etc.) vergeleken tussen de patiënten met glottische en supraglottische tumoren. Glottische tumorlokalisatie was significant vaker gerelateerd aan klinische kenmerken zoals het mannelijk geslacht, stemklachten als eerste symptoom, tumorstadium T1, de afwezigheid van uitzaaiingen naar lymfklieren en een hogere leeftijd. Supraglottische tumorlokalisatie was, in minder overtuigende mate, gerelateerd aan het vaker voorkomen van afstandsmetastasen na initiële diagnose/behandeling. In supraglottische tumoren was de mate van aanwezigheid van HIF-1a, Cortactin, EGFR en Ki-67 significant hoger dan in glottische tumoren. Anderzijds, de mate van aanwezigheid van CA-IX en Cyclin D1 was significant hoger in glottische tumoren.

Verschillen in de aanwezigheid van tumormarkers, de voorspellende waarde van tumormarkers en de klinische kenmerken tussen vroeg-stadium glottische en supraglottische tumoren suggereren dat

supraglottische en glottische tumoren als verschillende ziekten beschouwd zouden moeten worden, elk met zijn eigen afzonderlijk klinisch en biologisch gedrag. Dit zou ook een verklaring kunnen zijn voor de in de literatuur gerapporteerde verschillen in associaties van klinische uitkomstparameters, zoals lokale controle en overleving, en de verschillende tumormarkers in HNSCC. Onze bevindingen suggereren ook dat resultaten van studies die glottische en supraglottische tumoren evalueren zonder onderscheid te maken, met behoedzaamheid geïnterpreteerd dienen te worden. Derhalve raden wij voor toekomstig tumormarker-onderzoek aan supraglottische en glottische tumoren afzonderlijk te benaderen.

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Short biography

Jan Edward (Jasper) Wachters (born Emmen, The Netherlands, 1981) received his pre-university education (VWO) diploma in 1999 from the RSG Ter Apel, the Netherlands. In the year 2000 he started his university studies in Medicine at the University of Leiden, the Netherlands, receiving his medical degree in 2007, with a graduation thesis on stem cells and the in vitro cartilage development in the orthopedic clinic of the University of Heidelberg, Germany. In the period 1999-2007 Jasper had, from a clinical perspective, several evasive jobs in and outside the Netherlands (amongst others airport assistant for the disabled, parking garage manager, farmer, bicycle mechanic), before starting in 2007 as a basic medical doctor in the department of Otorhinolaryngology of the HagaZiekenhuis under the direction of dr. H.M. Blom, The Hague, The Netherlands. He started his otorhinolaryngological research activities at the department of Otorhinolaryngology and Head and Neck Surgery and the department of Pathology of the University Medical Center Groningen (UMCG), The Netherlands, in 2008 under the direction of prof. dr. E.M. Schuurung, prof. dr. B.F.A.M. van der Laan and dr. J.E. van der Wal. Starting in 2010, this work was combined with his residency in the department of Otorhinolaryngology and Head and Neck Surgery in the UMCG under the direction of prof. dr. B.F.A.M. van der Laan and in the Martini Hospital Groningen under the direction of dr. J.Q.P.J. Claessen and dr. J.R. de Boer. Since finishing his residency in 2015, he works as a member of staff of the UMCG, focussing on surgery of benign anomalies of the larynx and trachea. Jasper lives together with Anna Veenstra, psychologist, in Noordwolde (Groningen). They have three children, Isabel, Julia and Joe.

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