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Exploring and validating innovative methods for detection and localization of head and neck squamous cell carcinoma primary tumors and lymph node metastases

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General introduction
Scope of this thesis

General introduction

Head and neck cancer represents the seventh most common cancer worldwide, accounting for 890,000 cases and 450,000 deaths per year.[1] Most head and neck cancers arise from mucosal squamous epithelium in the oral cavity, oropharynx, hypopharynx, larynx, and nasopharynx. For these cancers, the collective term head and neck squamous cell carcinoma (HNSCC) is used. The two main risk factors are tobacco and alcohol use, which have a synergistic effect.[2,3] The human papillomavirus (HPV) is another cause for development of oropharyngeal squamous cell carcinoma (SCC), that is increasingly prevalent over the past decades, and is transmitted through sexual activity.[4] Nasopharyngeal SCC has a distinct Epstein-Barr virus (EBV)-related etiology with a relatively low prevalence in the Netherlands. Of HNSCC in the Netherlands in 2019, 34% arose in the oral cavity, 26% in the larynx, 26% in the oropharynx, and 9% in the hypopharynx.[5]

HNSCC can be classified as early-stage (I and II) in case of smaller tumors with no prominent nodal metastasis, or as advanced stage (III and IV) with larger tumors and regional or distant metastasis. As HNSCC usually gives little complaints in earlier stages, over 60% of patients present with stage III-IV disease at the time of diagnosis with limited treatment options and a poor 5-year prognosis of less than 50%.[6,7] As delay in time to treatment further worsens the prognosis,[8-10] timely, rapid and accurate diagnosis and a prompt start of treatment are essential.

The current diagnostic workup consists of physical examination and endoscopy by flexible laryngoscopy or the naked eye (**Fig. 1**). In case a malignancy is suspected, a biopsy of the primary tumor is taken to confirm the diagnosis by histopathological examination. Standard staining used for diagnosis is hematoxylin and eosin (H&E) (**Fig. 2**). Histomorphologically well-differentiated SCC is characterized by keratinization, horn pearls and the presence of intercellular bridges (desmosomes). Additional immunohistochemical stainings (e.g., CK5/6, p63 or p40) may be performed to confirm diagnosis in less differentiated SCC or to reveal additional information, such as p16 as a surrogate marker for HPV in oropharyngeal SCC, or epidermal growth factor receptor (EGFR), which is the target of systemic treatments like cetuximab.[6]

When an HNSCC has been confirmed histologically, conventional radiologic imaging such as computed tomography (CT) of the head, neck and thorax and magnetic resonance imaging (MRI) of the head and neck is performed to determine tumor stage (i.e., the extension of the primary tumor, the presence of lymph node and/or distant metastases). A CT-scan is often made initially as it is more readily available, faster, and cheaper compared to MRI.[11] Ultrasound (US) without or with US-guided fine-needle aspiration cytology (FNAC) is performed when lymph node metastasis is suspected. FNAC has an 82-92% sensitivity for the detection of HNSCC lymph node metastases.[12-14] The presence of lymph node metastasis determines the disease stage and choice of treatment to a great extent. It is therefore necessary to detect lymph node metastases as soon and accurately as possible.

Figure 1. Diagnostic process and treatment of head and neck squamous cell carcinoma, with the studied techniques and their respective chapters.
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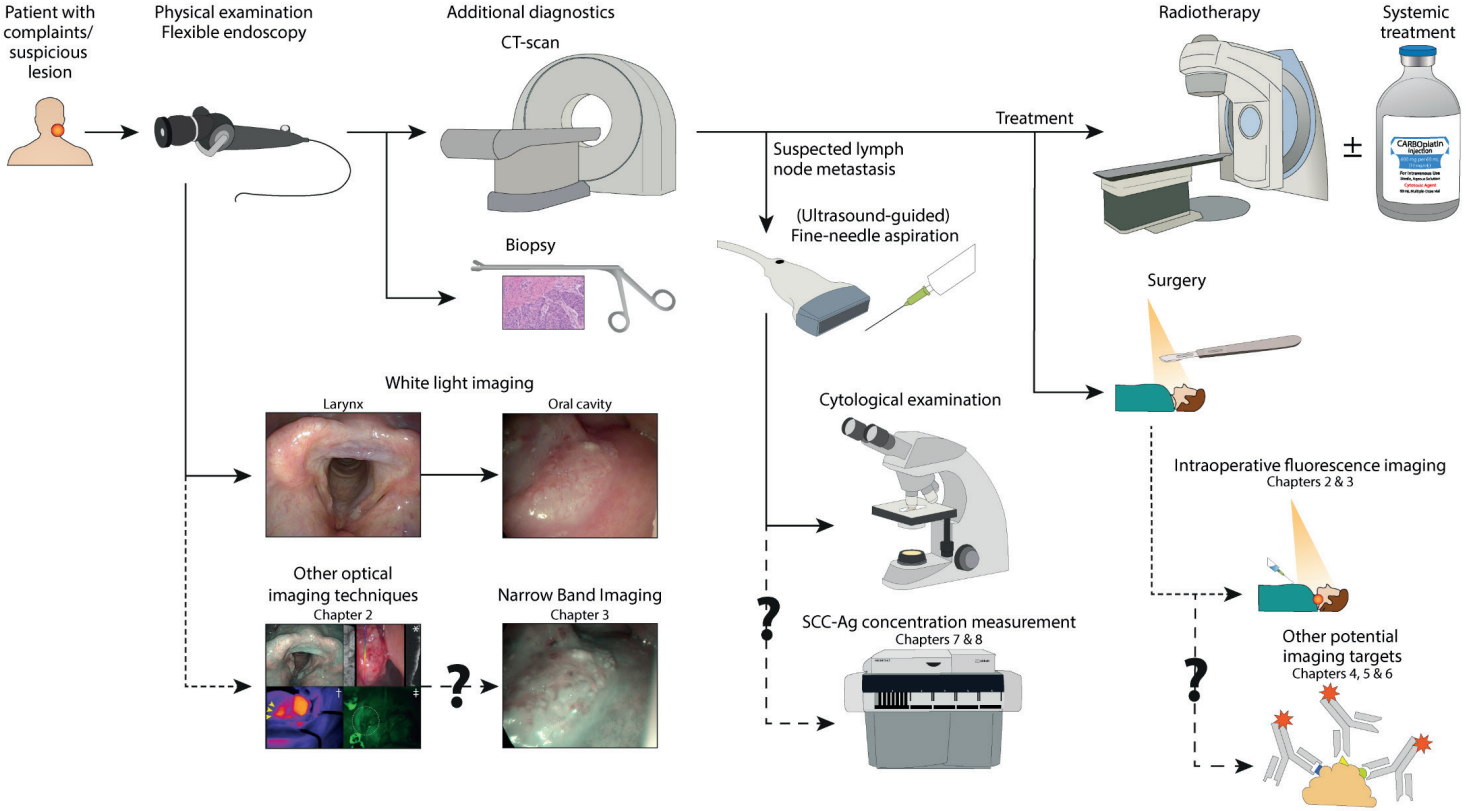
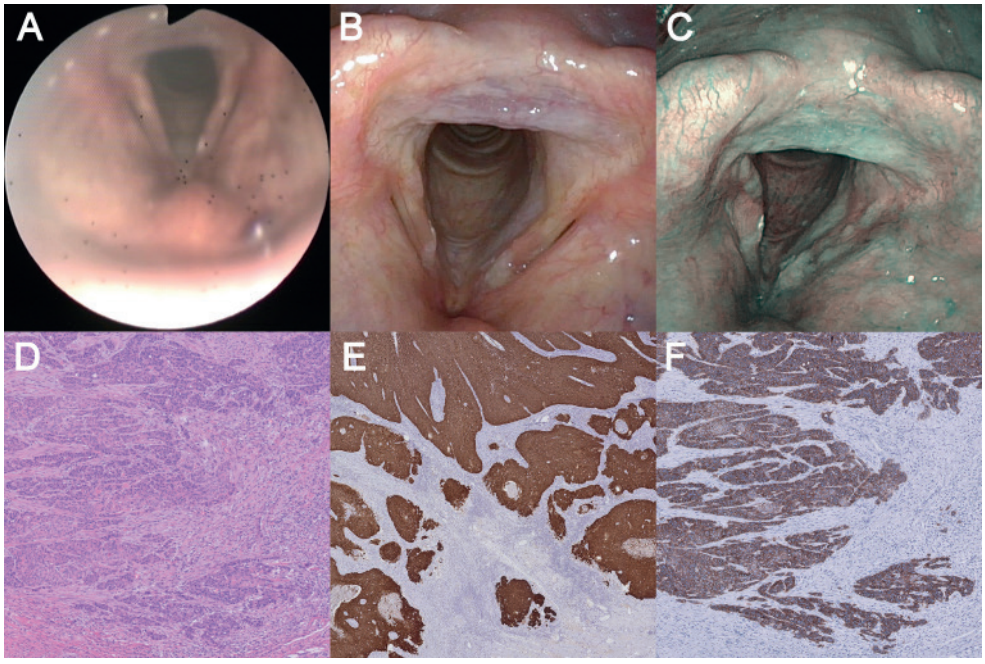


Depending on disease site, progression and the presence of regional metastasis, treatment may consist of surgery, radiotherapy, systemic therapy (i.e., chemotherapy, targeted immunotherapy) or a combination. For smaller lesions, surgical excision or radiotherapy may be the primary choice of treatment. In more advanced stages, a combination of treatment modalities is chosen, such as chemoradiotherapy or surgery with adjuvant (chemo) radiotherapy. The aim of surgical treatment is complete tumor removal, while maintaining function and cosmetics as well as possible. Since there are limited intraoperative techniques to guide the surgeon in this anatomically delicate and challenging area, tumor-positive surgical margins (<1 mm) still occur in for example up to 43% of oral SCC resections,[15] consequently leading to residual or recurrent disease. Recurrent or metastatic disease after initial treatment occurs in 65% of patients,[6] for which a second treatment period of irradiation or salvage surgery of the recurrent tumor is the last chance for curation. In salvage surgery, free surgical margins are difficult to achieve due to fibrosis after previous irradiation. Moreover, high complication rates up to 67% have been reported, and the 5-year survival is only 30-58%.[16,17] Patients who do not respond to or cannot receive this last treatment option have a prognosis of only 6 to 9 months.[6] Therefore, salvage surgery should never be a backup option, and curation and complete resection should always be strived for at the first attempt.

Figure 2. Various diagnostic techniques.
Flexible laryngoscopy: A) fiberoptic, B) High definition, C) High definition Narrow Band Imaging
Standard immunohistochemical stainings: D) hematoxylin & eosin, E) p16, F) Epidermal growth factor receptor.



Up to 4% of patients present with a lymph node metastasis of an unknown primary tumor (UPT).[18] In case an UPT is suspected after standard workup with physical examination and flexible endoscopy, a positron-emission tomography (PET)/CT will be made. If the primary tumor still remains unidentified, directed biopsies of the oropharynx, hypopharynx and nasopharynx may be taken with panendoscopy under general anesthesia. Since these primary tumors are mostly located in the palatine tonsil or base of tongue,[18] diagnostic tonsillectomy in combination with removal of lymphoid tissue of the base of the tongue (so called mucosectomy) is performed in search of the primary tumor. Despite this diagnostic workup, 40% of primary tumors are not found.[18]

New techniques, such as transoral laser microsurgery (TLM) and transoral robotic surgery (TORS) have improved visualization and maneuverability compared to traditional instrumentation.[19] Tactile feedback during surgery, important for the identification of tumor, is lacking with TLM/TORS. Therefore, better visualization using new intraoperative imaging techniques could aid in detection and localization of tumors, resulting better outcomes.

Early and rapid detection

For earlier diagnosis, improvements can be made at the first inspection. Technical developments of the past decades have improved the detection rate of HNSCC, such as improved CT/MRI image quality, and the use of high-definition rather than fiberoptic laryngoscopy, increasing the sensitivity for distinguishing benign from malignant lesions from 79.8% to 91.7%.[20] However, small superficial lesions can easily be missed by visual inspection or are too small to be detected by CT/MRI scan, and larger tumors may be missed due to submucosal extension.

The introduction of digital video processing for high-definition imaging, which uses charge-coupled devices on the tip of videoendoscopes, also allowed the addition of other techniques, such as narrow band imaging (NBI). NBI uses two bands of blue and green light, which are maximally absorbed by hemoglobin, and in this way can better visualize blood vessels. This can be used to recognize the abnormal blood vessel patterns that are typical in carcinogenesis, and lead to improved detection of HNSCC compared to conventional white light imaging.[21,22]

Near-infrared (NIR) fluorescence imaging can also be used for the detection and visualization of HNSCC. Exogenous fluorescent agents are injected and accumulate in the target tissue, which can be achieved by untargeted fluorescent agents due to general physiological changes in malignant tissues, or by targeted antibody-dye fluorescent agents that bind to tumor-specific receptors. The tumor can then be visualized by excitation of the fluorescent dye by certain wavelengths of light. The light is absorbed and subsequently emitted with slightly less energy at a longer wavelength, which can be captured by cameras. The NIR spectrum (700-900 nm) is the optimal window for fluorescence imaging due to minimal absorption by other components, which does occur at shorter and longer wavelengths, allowing the light to travel up to millimeters.[23] Fluorescence imaging has been applied successfully in the visualization of HNSCC, and led to the detection of unanticipated second primary lesions that would have been missed using conventional white light only.[24,25]

Another step in the diagnostic process is to determine the presence of regional lymph node metastasis. When a patient is suspected to have a lymph node metastasis on a CT or MRI scan, this has to be confirmed by FNAC. A radiologist locates the suspect lesion (e.g., enlarged or radiologically suspicious lymph node) under ultrasound guidance, punctures it with a fine needle, and aspirates cells. Several samples may be taken when multiple suspicious lymph nodes are encountered, however, due to the associated workload, usually only the most suspicious one or two lymph nodes are sampled. The pathology technician then performs a quick staining to check whether sufficient material has been harvested. If adequate, the material is subsequently prepared to be analyzed by a pathologist, and the result follows in the next days. However, sometimes still insufficient material has been collected for a diagnosis or the sample was contaminated by blood. FNAC is also applied in the diagnosis of other cancer types, such as breast and thyroid cancer.[26,27] Although FNAC has a high sensitivity of 82-92% and specificity of 97-100% for the detection of HNSCC lymph node metastases,[12-14] the process of cytological examination is labor intensive and costly and may take up to five working days until diagnosis. Histology remains the gold standard for diagnosis since this allows to evaluate cells in the context of the surrounding tissue. However, this requires a more invasive procedure, such as a biopsy. Serum tumor markers may also indicate disease and provide prognostic information. The squamous cell carcinoma antigen (SCC-Ag) is used as such a prognostic serum tumor marker in, for example, cervical cancer.[28,29] In HNSCC, serum SCC-Ag was associated with tumor and nodal stage, however, it was not with survival and can therefore not used as a predictive marker.[30] Measurement of tumor markers in fine-needle aspiration (FNA) samples is a new method that has not been described in the literature before. SCC-Ag concentration measurement in FNA samples could be used as a supportive tool for FNAC to more accurately diagnose SCC lymph node metastases, possibly leading to earlier diagnosis and reduced diagnostic turnaround time.

Intraoperative visualization

Currently, the surgeon is dependent on visual and tactile information to intra-operatively guide the resection, supported by preoperative radiologic imaging. In an attempt to reduce tumor-positive surgical margins (<1 mm), which occur in up to 43% of resections,[15] frozen section margin assessment is a widely used technique. Frozen section analysis is performed by taking a sample of the excised specimen at the (suspected) closest margin, after which it is frozen to -15°C to -30°C, cut into a 7 µm slice, fixated onto a glass slide, stained with H&E, and evaluated by a pathologist.[31] However, this technique is time-consuming, prone to sampling error, and subject to interobserver variation.[31] On the other hand, conventional imaging does not provide sufficient spatial resolution for intraoperative margin assessment, and is challenging to be used in the operating theater due to the related logistics and limited availability. Therefore, new techniques, such as NBI and NIR fluorescence imaging, could assist the surgeon during surgery in determining tumor margins by intraoperative real-time visualization and delineation of HNSCC.

NBI can only be used in vivo for the determination of mucosal margins before incision, since blood will obscure the operative field once the resection has started. Moreover, the

intraepithelial papillary capillary loops visualized by NBI, which are typical for malignancy, are only present in the submucosa and not in the deep tissue resection margin. Despite all this, first studies on the use of NBI for intraoperative margin assessment report promising results.[32-35]

NIR fluorescence imaging can be used for both mucosal and deep surgical margin assessment. Since targeted fluorescent imaging can be used both in vivo and ex vivo, as the fluorescent agents remain bound to the receptor, margin assessment can also be done on the freshly excised specimen by fluorescence imaging. Margin assessment by fluorescent imaging only takes minutes and is not prone to sampling error, and could therefore substitute frozen section assessment.

Since both techniques can be applied for the visualization of superficial mucosal lesions, studies are required to investigate which technique performs best in mucosal margin assessment.

Potential targets for NIR fluorescence imaging

Currently, EGFR-targeting fluorescent agents are used for NIR fluorescence imaging in HNSCC. While EGFR is expressed in 87.5-92.5% of primary HNSCC tumors,[36-38] fluorescent EGFR-signal has also been encountered in surrounding normal tissues. Other proteins expressed in HNSCC may potentially serve as a target for imaging, such as vascular endothelial growth factor (VEGF), which is expressed in 87.5-95.0% of HNSCC.[37,38] VEGF is often highly expressed in tumors, as it stimulates angiogenesis, which is required to meet the higher oxygen demand as a result of an altered metabolism in cancer cells. Another potential target, recently identified by a method analyzing mRNA expression data, is glycoprotein non-metastatic melanoma type B (GPNMB), with expression seen in 92% of HNSCC.[39] GPNMB was first described in a cell line of melanoma with low potential for metastasis,[40] and a positive correlation between GPNMB expression and TNM-stage has been described in HNSCC.[41] For VEGF, the VEGF-targeting fluorescent probe bevacizumab-800CW has already been applied successfully in other cancer types.[42-46] Since there are clinically approved antibodies available for GPNMB, a fluorescent probe could be produced relatively easily. These targets could be more sensitive and specific than EGFR, and lead to higher contrast between tumor and background tissue (tumor-to-background ratio; TBR) when applied in HNSCC, resulting in better detection and consequently better surgical outcomes. Therefore, potential also lies in the identification of other targets for fluorescence imaging in HNSCC, and as a first step, expression of GPNMB and VEGF needs to be assessed in comparison to EGFR expression.

A new method that can be applied for the identification of potential imaging targets is transcriptional adaptation to copy number alterations (TACNA) profiling.[47] This biostatistical method captures downstream effects of copy number alterations by using publicly available transcriptomic data. In this manner, proteins overexpressed in HNSCC in comparison with healthy mucosa of the head and neck region can be identified. This could lead to potential targets for fluorescence imaging.

Outline of this thesis

A first step to improve clinical outcome of HNSCC is to improve the visualization and thereby the detection and localization of primary HNSCC, in order to ensure an earlier diagnosis and to improve surgical outcome. New techniques show promising results that could aid in the detection and visualization of HNSCC and their lymph node metastases. The aim of this thesis was to investigate NBI and fluorescence imaging for the detection and visualization of primary HNSCC, to evaluate potential new imaging targets to improve the accuracy of intraoperative visualization of HNSCC, and to determine the diagnostic value of protein concentration measurement in FNA samples for the detection of HNSCC lymph node metastases.

Chapter 2 is a review on various optical techniques for better intraoperative visualization and detection of HNSCC. In this chapter, a broader insight is provided on the background, working mechanisms and clinical results achieved thus far by these optical techniques. The review mainly focuses on the diagnostic and intraoperative application of NBI and NIR fluorescence imaging.

To determine which of these two techniques offers the most reliable assessment of mucosal margins, we conducted a pilot study that is described in **chapter 3**. In this prospective study we compared assessment of the mucosal tumor margin in oral cavity carcinomas by applying both NBI and NIR fluorescence imaging using cetuximab-800CW. The results of the mucosal tumor margin assessment from both techniques were compared ex vivo to histopathology results.

Fluorescence imaging studies in HNSCC are currently targeted at the epidermal growth factor receptor (EGFR). However, other targets could be more specific for HNSCC and result in higher TBRs in fluorescence imaging. In **chapter 4** the search for new, more specific targets for fluorescence imaging of HNSCC primary tumors is described. We used a biostatistical method called transcriptional adaption to copy number alterations (TACNA) profiling to translate publicly available mRNA data to expression of HNSCC specific cell membrane located proteins. Results were immunohistochemically validated on biopsies of tumor tissue, adjacent suspicious tissue, and normal mucosa. However, clinically approved antibodies for these targets are not available yet. Therefore, we evaluated the expression of known targets for HNSCC other than EGFR as potential new targets fluorescence imaging in **chapter 5**. Using immunohistochemistry, we evaluated expression of vascular endothelial growth factor (VEGF) and glycoprotein non-metastatic melanoma type B (GPNMB), compared with EGFR, in previously untreated primary HNSCC tumors and corresponding lymph node metastases.

Since patients with residual or recurrent disease after (chemo)radiotherapy have an especially dire prognosis and would benefit greatly of improved detection, we evaluated whether expression changes after previous treatment, for the purpose of fluorescence imaging. In **chapter 6** we therefore compared expression of GPNMB and VEGF with EGFR as potential fluorescence imaging targets in the detection of HNSCC lymph node metastasis after initial (chemo)radiotherapy.

Besides the detection of primary tumors, accurate diagnosis of regional lymph node metastasis is important for staging and choice of treatment. To explore a new technique of biomarker concentration measurement for the diagnosis of HNSCC lymph node metastases,

we measured SCC-Ag in cervical FNA samples to determine its diagnostic value. This retrospective pilot study is described in **chapter 7**. A prospective study to establish the diagnostic value of SCC-Ag and cancer antigen (CA) 15-3 as a new technique for the detection of HNSCC lymph node metastasis is described in **chapter 8**.

Chapter 9 contains a summary and general discussion.

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