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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Summary and general discussion

Over 60% of head and neck squamous cell carcinoma (HNSCC) patients present with locally advanced disease, which is still associated with a poor 5-year survival of less than 50%. The prognosis is further worsened by delays in time to treatment and inadequate surgical margins (<5 mm). The aim of this thesis was to investigate Narrow Band Imaging (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. This could subsequently lead to both a more rapid diagnosis and fewer tumor-positive surgical resection margins.

In chapter 2 we reviewed several optical techniques for improved intraoperative real-time visualization and detection of HNSCC. NBI and near-infrared (NIR) fluorescence imaging were described in more detail since they provide wide-field real-time visualization of the tumor and have been gaining increased clinical interest. NBI uses blue and green light to visualize mucosal and submucosal blood vessels, which show aberrant patterns in malignancy. NBI performs better than white light imaging and has an 89% sensitivity and 96% specificity for the detection of HNSCC. Moreover, it can be used in the search for unknown primary tumors (UPT) with 74-91% sensitivity and 86-95% specificity rates, compared with only 44% and 97%, respectively, for PET-CT. NIR fluorescence imaging can be applied using untargeted agents, such as indocyanine green (ICG), which make use of the enhanced permeability and retention effect to accumulate in tumors. However, targeted agents using fluorophores conjugated to an antibody show better results. Using epidermal growth factor receptor (EGFR)-targeted agents to visualize and detect HNSCC, a 100% sensitivity and 74-91% specificity can be reached in a closed-field setting on the resected specimen. Additionally, fluorescence imaging can detect satellite tumor lesions that would otherwise have been missed, and can guide frozen section sampling to reduce sampling errors.

In order to determine which of these two techniques performs best in intraoperative mucosal margin assessment, we compared both techniques in oral SCC in chapter 3. NBI defined tumor borders were marked in vivo with three sutures, to compare them ex vivo with fluorescence imaging using EGFR-targeted cetuximab-800CW. In final histopathology, fluorescence imaging appeared to be more accurate in delineating the tumor border than NBI, with borders more frequently determined <1 mm (53% vs 13%, respectively), and <5 mm (97% vs 74%, respectively) of the histopathological tumor border. For fluorescence imaging, this is in line with findings using panitumumab-800CW, where 95-100% sensitivity was reported for the detection of tumor <5 mm of the surgical margin. We found that NBI mainly performs poorly in locally advanced tumors with submucosal extension, beyond the penetration depth of 240 µm of NBI. Therefore, NBI appears to be a good alternative to fluorescence imaging in early-stage tumors without submucosal extension. Indeed, first reports on the use of NBI to intraoperatively determine resection margins show improved outcomes compared to using conventional white light. In oral SCC, a five-year disease-free survival of 84% and local...
Summary, general discussion, and future perspectives

Adequate intraoperative visualization using fluorescence imaging relies on contrast between tumor tissue and normal surrounding tissue, which is defined by the tumor-to-background ratio (TBR). Fluorescence imaging studies in HNSCC have focused on targeting EGFR, which is expressed in 90% of HNSCC primary tumors, and resulted in an in vivo TBR of 2.5 and ex vivo TBRs of 3.1-6.5. In vivo imaging results are affected by several factors, such as ambient light, camera angle, and the distance between the camera and the patient. Fluorescent signal of EGFR-targeted agents has also been noted in basal and suprabasal epithelial cells and in salivary gland tissue. Therefore, more specific targets for HNSCC could further improve in vivo TBRs and make image-guided surgery feasible, rather than ex vivo imaging on the freshly excised specimen.

In chapter 4 we aimed to identify potential targets for fluorescence imaging in HNSCC using a biostatistical method known as Transcriptional Adaptation to Copy Number Alterations (TACNA) profiling. TACNA profiling captures the downstream effects of copy number alterations (CNAs) on mRNA level, and can be used to predict overexpression on protein level. Targets were selected based on the degree of overexpression, plasma membrane expression and cross expression in other tissues that may be relevant in head and neck surgery. In this manner, we found four targets: glucose transporter 1 (GLUT-1), placental cadherin (P-cadherin), monocarboxylate transporter 1 (MCT-1), and neural/glial antigen 2 (NG2). These findings on mRNA level were validated on protein level by immunohistochemistry (IHC) in biopsies of HNSCC, adjacent suspicious tissue, and of normal mucosa. The targets showing the highest expression were GLUT-1 and P-cadherin, both expressed in all tumors and with the highest median percentage of expression in tumor cells (100%). GLUT-1 is the main glucose transporter that is overexpressed to meet the higher glucose demand in cancer cells, and our results are in line with the literature describing expression in 96-100% of oral cavity tumors. P-cadherin is a cell-cell adhesion glycoprotein that had higher expression than healthy mucosa in a laryngeal SCC cell line microarray. We found the percentage of expression in tumor cells to be significantly higher for GLUT-1 and P-cadherin compared with the percentage of EGFR. Based on these findings by IHC, GLUT-1 and P-cadherin are promising targets for fluorescence imaging in HNSCC as an alternative to EGFR. However, GLUT-1 expression was also seen in erythrocytes, and P-cadherin expression was seen in the basal layer of the mucosa, which could reduce the TBR during fluorescence imaging. There are no clinically approved antibodies available for these targets yet.

In the search for potentially better HNSCC fluorescence imaging targets than EGFR, we compared EGFR expression with expression of targets for which already exist clinically
approved antibodies, namely glycoprotein nonmetastatic melanoma protein B (GPNMB), and vascular endothelial growth factor (VEGF), for which exists the fluorescent probe bevacizumab-800CW. In chapter 5 we evaluated GPNMB and VEGF expression, in comparison to EGFR as a traditional marker, in untreated primary tumors and corresponding lymph node metastases. We found the highest expression for GPNMB, which was expressed in all primary tumors and lymph node metastases. GPNMB also showed expression in a significantly higher percentage of tumors cells in both primary tumors and lymph node metastases than EGFR. VEGF was expressed in a comparable number of tumors (92.1%) as EGFR (86.8%), both concordant with the literature, which reports 87.5-95.0% for VEGF and 87.5-92.5% for EGFR. The high expression of GPNMB could result in higher TBRs in intraoperative fluorescence imaging. Moreover, due to the simultaneous expression in primary tumors and lymph node metastases, one can be sure that if a biopsy of the primary tumor is positive for GPNMB, the lymph nodes will also be positive. Conversely, in case a lymph node metastasis of a (yet) UPT is positive for GPNMB, fluorescence imaging can be applied in the search for the primary tumor.

The resection of any recurrent or regional metastatic disease is especially crucial in patients who have previously received (chemo)radiotherapy. For them, the 5-year survival is 30-58% and salvage surgery is the last treatment option with a chance for cure. The prognosis in case of recurrent disease after failure of treatment by salvage surgery or radiotherapy is only six to nine months. In chapter 6 we assessed the expression of GPNMB, VEGF, and EGFR in lymph node metastases after initial (chemo)radiotherapy to identify potential targets for fluorescence imaging and compared lymph nodes with and without viable metastatic tumor. We compared expression of GPNMB and VEGF with EGFR, as the traditional marker, in lymph nodes with viable tumor metastases, reactive changes as a result of (chemo)radiotherapy (e.g., necrosis), and normal lymph nodes. GPNMB was expressed in all lymph node metastases, while EGFR was expressed in only 86% of lymph node metastases, similar to our findings in chapter 5 and those in literature for EGFR. Expression of both targets was also seen in necrosis, possibly confounding fluorescence imaging results in vivo. VEGF was highly expressed in all lymph node metastases, lymph nodes with reactive changes, and normal lymph nodes. Therefore, based on our IHC results, GPNMB appeared as a promising target for fluorescence imaging at least comparable to EGFR, while VEGF seemed unsuitable after initial (chemo)radiotherapy.

However, IHC results cannot be compared directly to in vivo imaging results. While a sensitivity rate of 100% for HNSCC has been described for in vivo EGFR-targeted fluorescence imaging, the literature describes EGFR expression in only 87.5-92.5% of HNSCC. This difference may be attributed to the in vivo receptor activity of EGFR, which cannot be determined by IHC. Moreover, while false-positive signal has been reported in VEGF-targeted fluorescence imaging of soft-tissue sarcomas after neoadjuvant radiotherapy, VEGFA targeted fluorescence endoscopy has also been applied successfully in rectal cancer after radiotherapy. This underlines the need to validate our IHC findings of GPNMB in a preclinical model or by topical application on a freshly excised HNSCC specimen as the next step towards in vivo fluorescence imaging.
Besides the primary tumor, the early and accurate detection of HNSCC lymph node metastases is important for staging, choice of treatment and prognosis. In case of suspected lymph node metastasis, fine-needle aspiration (FNA) is performed with subsequent cytological examination. Fine-needle aspiration cytology (FNAC) has a sensitivity of 82-92% and specificity of 97-100% for the diagnosis of HNSCC lymph node metastases. However, it is a costly and labor-intensive procedure. In cervical cancer, squamous cell antigen (SCC-Ag) serves as a prognostic serum tumor marker. In HNSCC, however, serum SCC-Ag did not show any relation to metastasis or overall survival. SCC-Ag had never been measured in FNA samples before, and we hypothesized this could serve as an objective tool to diagnose HNSCC lymph node metastases with results available within 2 hours. In chapter 7 we conducted a retrospective pilot study in patients with neck masses, not necessarily suspicious for metastatic disease, to determine the diagnostic value of SCC-Ag in FNA samples of the neck. We found the most optimal cutoff concentration for SCC-Ag to be at 0.3 µg/L, which resulted in a 95.8% sensitivity and 74.4% specificity for the detection of HNSCC lymph node metastases. Although SCC-Ag had higher sensitivity for HNSCC than FNAC, the specificity was relatively low and would result in many false-positive results. We therefore hypothesized that SCC-Ag could serve as a screening tool prior to FNAC, to select lymph nodes suspect of having HNSCC metastases for subsequent cytological examination, to reduce workload for the pathologist and speed up the diagnostic process. Moreover, we demonstrated that biomarker measurement in FNA samples is technically possible and feasible for diagnostic purposes.

To prospectively establish the diagnostic value of SCC-Ag in FNA samples in patients with neck masses clinically suspicious for metastatic disease, we performed the study described in chapter 8. We added the measurement of cancer antigen (CA) 15-3 since in the retrospective study, branchial cleft cysts showed high SCC-Ag concentrations and resulted in false-positives. CA15-3 is the soluble form of Mucin 1, and mucins are typically present on the apical surface of glandular or luminal epithelial cells. Therefore, we hypothesized additional CA15-3 measurement could reduce false-positive results. At an SCC-Ag cutoff concentration of ≥0.1 µg/L, we found an 89.4% sensitivity and a 79.3% specificity for the detection of SCC metastases in cervical lymph nodes. Although the median concentration of CA15-3 was higher in cystic lesions, addition of CA15-3 after SCC-Ag measurement did not lead to improved accuracy for the detection of SCC lymph node metastases. In conclusion, SCC-Ag has a similar sensitivity and lower specificity compared to FNAC. Moreover, it does not lead to a classifying diagnosis. SCC-Ag as a screening tool prior to cytological examination does, therefore, not seem feasible. It could serve as a cheap and easy additional tool in FNA samples with a nondiagnostic or indeterminate cytological result, which occur in 2.6-9.6% of cases, since SCC-Ag can still be measured. In four cases of SCC metastasis, SCC-Ag was positive while cytology was false-negative. SCC-Ag could have indicated that these patients required timelier and closer follow-up. Moreover, SCC-Ag could aid in staging the disease. FNA samples can be taken from multiple enlarged lymph nodes, and after confirmation of SCC lymph node metastasis in the largest or most suspect lymph node, the other (e.g., contralateral) lymph nodes can easily and cheaply be screened using SCC-Ag.
While FNAC in cervical lymph node metastases has been reported to have high sensitivity rates up to 92%, it is not as sensitive in lesions of the salivary gland, with a 78% sensitivity reported for parotid lesions and 71.6% for submandibular lesions.\textsuperscript{49,50} If similar sensitivity rates of SCC-Ag for SCC could be reached in FNA samples of the salivary glands, it could prove to be a useful tool.

**Future perspectives**

Clinical application after further development of the techniques described in this thesis could improve the outcome and prognosis of HNSCC, as has already been described for intraoperative NBI and fluorescence imaging. Improvement of these techniques, or the development of complementary tools, could further increase detection rates and improve visualization. Like any new technique these have to follow the adoption cycle of new techniques, which start with innovators and early adopters before these new techniques are accepted by the majority of clinicians after thorough scientific evidence.\textsuperscript{51} The studies described in this thesis are the first steps in this adoption cycle of new techniques.

In recent years, the development of artificial intelligence has led to new possibilities for rapid, standardized, and objective analysis of data. These algorithms can also be developed to distinguish benign from malignant disease. Since a current system is already able to analyze up to 50 NBI images per second, it can be applied for real-time analysis.\textsuperscript{52} Moreover, by pointing out abnormalities, artificial intelligence could aid less experienced observers, such as residents, and reduce their learning curve for NBI. So called neural networks are based on the behavior of the human brain and can be used for pattern recognition. A training dataset with for example normal mucosa and HNSCC can be used to allow for the neural network to recognize patterns, and the bigger the size of the training dataset, the better the neural network will function. First attempts at using neural networks in the detection of oropharyngeal SCC using NBI were able to reach 71-86% sensitivity and 59-69% specificity, compared to 47-70% sensitivity found for white light imaging.\textsuperscript{52,53} A neural network developed to distinguish laryngeal papilloma and carcinoma from healthy vocal cords reached a 94% sensitivity and 91% specificity.\textsuperscript{54} The accuracy of neural networks is highly dependent on the size of the training dataset and the information that is considered, such as color and textural information, and blood vessel characteristics such as growth patterns and width. Moreover, proper visualization of the lesion is required for optimal analysis. By standardizing the visualization procedure and by adding more features to be analyzed by the neural networks, artificial intelligence has the potential to beat the human eye.

For fluorescence imaging, developments in several aspects could further increase TBRs or improve in vivo imaging, resulting in higher accuracy. Progress can be made such as the most tumor specific target, the fluorescent agent, the visualization of the fluorescence signal, and the interpretation of the signal. Developments could focus on improved accumulation of the fluorescent agent in the target tissue by aiming at targets more specific for HNSCC. Our translation of publicly available mRNA expression data by use of TACNA analysis to
analysis of protein expression of selected targets in HNSCC opens doors for new imaging targets. The targets GLUT-1, P-cadherin, and GPNMB described in chapters 4, 5, and 6 showed promising results with immunohistochemical expression higher than EGFR. Towards new targeted molecular imaging agents, antibodies for GLUT-1 and P-cadherin will have to be developed and eventually approved for clinical use. Once the antibodies have been developed, fluorescently labeled probes can be produced by conjugating a fluorescent dye to the antibody. This step could already be performed for GPNMB. At the University Medical Center Groningen, we have the facilities to produce these fluorescent probes. They can then be applied in vivo in xenograft models or be topically applied ex vivo on a freshly excised HNSCC specimen in small pilot studies, to assess the correlation with the IHC results found in chapters 4, 5, and 6, and to determine the feasibility of their application as fluorescent probes.\textsuperscript{35,36} Subsequently, after approval for clinical use, the safety profile has to be determined in a phase-I dose escalation study.

New developments may also be expected in the type of targeting agent. Currently, whole antibodies are used, such as cetuximab and bevacizumab. However, they could lead to relatively high background signal due to their large hydrodynamic diameter, resulting in liver accumulation, a longer bloodstream half-life, and slower clearance from the blood.\textsuperscript{19,55} The use of smaller antigen-binding agents has the potential to overcome this problem, such as nanobodies and affibodies. Nanobodies are derived from antibodies, but are only one tenth of the molecular weight of monoclonal antibodies, as a result of which they can more easily extravasate and are more rapidly cleared.\textsuperscript{35,56} Affibodies are engineered peptides. Due to their small size, they distribute rapidly, allowing for same-day surgery only hours after injection, and clear more rapidly, reducing risk for toxicity compared to monoclonal antibodies.\textsuperscript{57} Quantum dots are another type of promising agents that have a very small diameter of only 2-10 nm.\textsuperscript{58} Their excitation and emission wavelengths can be tuned by changing the composition and size, and they are typically brighter, have longer photostability, and longer fluorescence lifetime than organic dyes.\textsuperscript{59} However, since the core of quantum dots are typically made up of heavy metals and are toxic when released, clinical application may have to wait until development of non-toxic cores or quantum dots with faster excretion.\textsuperscript{60} Fluorescent agents can also be engineered to be activated on-site by tumor specific characteristics or enzymes present in the tumor microenvironment. Tumors are known to have an acidic environment due to an altered metabolism, known as the Warburg effect. This characteristic can be exploited by pH-activatable agents that release the fluorophore in acidic environments, such as ONM-100, which has first in vivo results of 100% sensitivity and 57% specificity for the detection of HNSCC.\textsuperscript{61} Another strategy is the use of enzyme activity, where fluorescence is self-quenched until activated by enzymes such as matrix metalloproteases, which are often overexpressed in cancer cells and secreted into the tumor microenvironment.\textsuperscript{62}

Until now fluorescence imaging has been directed at the traditional near infrared wavelengths (NIR-I 700-900 nm), which offer tissue penetration up to millimeters and relatively low interference of other tissues compared to the visible light spectrum. Recent developments have, however, overcome the lack of long-wavelength cameras and biocompatible molecules, allowing for fluorescence imaging in the second biological window (NIR-II, 1000-1700 nm).\textsuperscript{62}
Fluorescence imaging in the NIR-II window has greater penetration depth and higher resolution, and could lead to more accurate tumor detection once functional probes have been developed.

As with NBI, optimal conditions are required for proper visualization of the tumor and its surrounding tissue. The distance between camera and patient, camera angle, and ambient light all negatively affect the visualization. For this reason, a closed-field setting with standardized conditions result in the highest TBRs. The hindrance of ambient light in vivo could be overcome by new image acquisition techniques adjusted to the frequency of the background lighting, resulting in images appearing as if they were acquired in a dark room. Other developments could be aimed at standardization of in vivo imaging, acquiring higher spatial resolution images, and 3D imaging to be able to also assess depth. Fluorescence imaging tumor borders are often determined manually by an experienced analyst. Artificial intelligence could analyze fluorescence imaging data in a standardized and objective manner, and do it more rapidly. Moreover, it does not require the analyst for intraoperative surgical margin assessment. The implementation of artificial intelligence for fluorescence imaging analysis could therefore lead to better outcomes at lower costs.

A new potential field for application of fluorescence imaging is transoral robotic surgery (TORS). Robotic surgery is mainly used for carcinomas in the oropharynx, hypopharynx, and larynx, and mostly performed with the da Vinci Surgical System. Since tactile feedback is lacking during robotic surgery, surgeons fully rely on visual information. Tumor-positive resection margins occur in 9.9-20.2% of cases, and could be reduced by improved intraoperative tumor visualization. First studies show that it is feasible to integrate a fluorescent camera into the da Vinci Surgical System and detect (auto)fluorescence. However, the use of the untargeted dye ICG in the detection of oropharyngeal SCC did not lead to any contrast between cancerous and non-cancerous tissue, possibly owing to the high vascularity in the oropharyngeal mucosa. Targeted fluorescence imaging during TORS may overcome this issue and lead to improved tumor visualization, consequently resulting in reduced tumor-positive surgical margins and higher UPT detection. Future studies could be directed at using cetuximab-800CW, available in our institute, with an integrated fluorescent camera in the da Vinci Surgical System for the intraoperative visualization of HNSCC or UPTs. First of all, a small feasibility study with a limited number of patients should be conducted. In case of promising results, a larger study can be performed to confirm earlier findings.

Another promising technique is optoacoustic (or photoacoustic) imaging. Upon a nanosecond pulsed (<10 ns) laser illumination, the sample tissue absorbs the light and converts it into heat, causing a thermal expansion and relaxation process. This consequently generates acoustic waves, which can be detected by ultrasonic transducers. Imaging depths up to 4 cm can be reached in vivo since sound scatters 1000 times less than light. Optoacoustic imaging can be used to visualize endogenous contrast agents, such as water, lipids, hemoglobin, and melanin. However, for better contrast it may be needed to use exogenous contrast agents. Clinically approved contrast agents, such as ICG, have drawbacks such as fast clearance from the body and poor aqueous stability. Therefore, there remains a need for optoacoustic imaging contrast agents with good biocompatibility and clearance profiles.
For the detection of lymph node metastases, we have shown that protein concentration measurement in FNA samples is technically possible and feasible for the detection of different types of lesions. However, we have chosen to measure SCC-Ag and CA15-3 based on assumptions, rather than on previous data or pilot studies. Future studies could be directed at evaluating concentrations of various other proteins at random or based on assumptions, or could perform an analysis directed at protein expression such as the TACNA profiling described in chapter 4, in order to find more accurate biomarkers for the detection of lesions in the neck. While we had primarily focused on the detection of SCC lymph node metastases, future studies can be aimed at finding more specific proteins for other benign or malignant lesions of the neck and possibly also of the salivary glands. In a pilot study, a selection of retrospectively collected FNA samples stored in Cytolyt can be analyzed to assess the feasibility of the given protein for a certain diagnosis. The number of measurements that can be done on one FNA sample still has to be determined, since this also affects the number of proteins that can be evaluated on one FNA sample in future studies. Based on the results of the proteins tested in the pilot studies, larger retrospective/prospective studies may be initiated to reliably establish the diagnostic value and sensitivity and specificity rates.

In the present studies, SCC-Ag and CA15-3 proved especially useful in indeterminate and nondiagnostic FNAC samples. To further elucidate the clinical value in these cases, a prospective study has to be commenced collecting all FNA samples of the neck and salivary glands in Cytolyt. When after cytological examination no reliable diagnosis can be determined, the SCC-Ag and CA15-3 concentrations can be measured in these samples specifically. The results will then have to be compared with the follow-up FNAC result or final histology to fully establish the diagnostic value. Due to the low incidence of indeterminate or nondiagnostic results, the study should preferably be performed in a multicenter setting.

With increased sensitivity and specificity, biomarker measurement in FNA samples could be an alternative to FNAC and lead to earlier diagnosis.
References


