Detection of Oral Squamous Cell Carcinoma and Cervical Lymph Node Metastasis Using Activatable Near-Infrared Fluorescence Agents

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Objective: To assess the feasibility of optical imaging using activatable near-infrared fluorescence (NIRF) agents to detect oral cancer and cervical lymph node metastasis in vivo.

Design: In vivo study.

Setting: University medical center.

Subjects: Female nude mice aged 4 to 6 weeks.

Intervention: Luciferase-expressing OSC-19-luc cells were injected into the tongues of nude mice. A control group of nude mice was injected in the tongue with a physiologic saline solution. Tumor growth was followed by bioluminescence imaging. After 3 weeks, animals were randomly allocated to intravenous administration of 1 of 2 activatable NIRF agents: ProSense680 or MMPSense680. Fluorescence imaging of the mice was performed, and the tumor to background ratio (TBR) was determined on histologic sections of the tongue and cervical lymph nodes after resection at necropsy.

Main Outcome Measure: Fluorescence signals.

Results: The fluorescence signals in tongue tumor and cervical lymph node metastases were significantly higher than those in control animals. The mean (SD) TBR of ProSense680 in the tongue was 15.8 (8.1) and in the lymph nodes was 11.8 (3.6). For MMPSense680, the mean (SD) TBR in the tongue was 18.6 (9.4) and in the lymph nodes was 10.5 (4.0).

Conclusions: Oral cancer and cervical lymph node metastases can be detected by targeting increased proteolytic activity at the tumor borders using NIRF optical imaging. These NIRF agents could be used for real-time image-guided surgery, which has the potential to improve the complete surgical resection of oral cancer.


Intraoperative Assessment of tumor-free margins in head and neck cancer surgery is critical to completely remove the primary tumor and improve prognosis while minimizing surgical morbidity. Currently, preoperative assessment of tumor size and involvement of regional lymph nodes is performed using different imaging modalities. However, in the operating room, the surgeon is confined to visual appearance and palpation of the tumor and cervical lymph nodes. As a result, involved surgical margins have been described in 16% of clinically radically resected oral and oropharyngeal squamous cell carcinoma (OSCC) specimens. Postoperative radiotherapy is often administered to decrease the local recurrence rate. However, local tumor recurrence for tongue carcinomas after curative intended surgery has been described in 22% of patients. Therefore, new intraoperative visualization techniques are required to assess tumor margins in real time and to guide the subsequent surgical removal with adequate tumor-free margins. Optical imaging using near-infrared fluorescence (NIRF) light has recently emerged as a promising technique that has the potential to traverse the gap between preoperative radiology and surgery by providing real-time visualization of tumor tissue, warranting image-guided surgery. The features of NIRF light provide crucial advantages for its application in image-guided surgery due to light absorption by hemoglobin in the visible light spectrum (<650 nm) and by other components, such as water and lipids, in the infrared range (>900 nm). This results in an optical imaging window from approximately 650 to 900 nm in which light scattering and non-specific autofluorescence are minimal. Furthermore, the absorption coefficient is low in this spectrum, resulting in an increase in tissue penetration, and interference of fluorescence with the surgical field is prevented due to the insensitivity of human eyes to near-infrared wavelengths.
When targeting specific cancer cells, a variety of strategies can be chosen that each target different tumor characteristics. To gain optimal tumor to background ratios (TBRs), new agents have been designed that contain a cleavage site specific to tumor-specific enzymes. These agents are injected in a quenched (ie, nonfluorescent) state, minimizing fluorescence at the time of administration. After cleavage by the specific enzyme, the agent becomes de-quenched (ie, fluorescent). Next to increased TBR, the use of activatable agents has a second specific advantage because they detect proteases that are associated with specific characteristics, for example, the invasive, aggressive, or metastatic tendency of the tumor.

The capability to degrade the extracellular matrix (ECM) surrounding the tumor to access blood vessels and lymphatic vessels is a prerequisite for tumors to invade and metastasize. In this process, the same mechanisms that normal cells use for migrating through tissue barriers are exploited. Proteolytic enzymes (including matrix metalloproteinases [MMPs] and cathepsins (mostly cathepsin B) are involved in ECM degradation of OSCC. Increased production of these enzymes has been associated with the invasive and metastatic phenotype of the tumor. Tumor-associated MMP expression and activity is the result of endogenous tumor cell expression. However, in epithelial cancers, most of the upregulated MMPs are expressed by host stromal cells surrounding the tumor, especially in areas of active invasion. Cathepsin B is mostly upregulated in lysosomes of tumor cells located at the invasive tumor border, where ECM degradation takes place and, hence, proteolytic activity is increased. Furthermore, cathepsin D has been described as a potential independent predictor of cervical lymph node metastasis in head and neck cancer. Therefore, NIRF agents that can detect these proteases may be useful for intraoperative detection of OSCC.

We performed a study to assess the feasibility of protease-activatable optical imaging for the detection of OSCC and cervical lymph node metastasis. To our knowledge, this is the first study to describe the use of activatable NIRF agents to detect OSCC in vivo.

METHODS

REAGENTS

The NIRF agent ProSense680 (VisEn Medical, Woburn, Massachusetts), with peak excitation at 680 nm and emission at 700 nm, was used for imaging of the tumor and involved margins. ProSense680 is conjugated to the fluorochrome VivoTagS680 (VisEn Medical) and becomes activated after cleavage by proteolytic activation of lysosomal cysteine or serine proteases, including cathepsin B. The second agent used was MMPSense680 (VisEn Medical), which is conjugated to the same fluorochrome and has the same fluorescence characteristics. This agent becomes activated after cleavage by MMPs.

CELL LINE

For this study, the human OSCC cell line OSC-19-luc was used, which is known to metastasize to cervical lymph nodes. This cell line was established in Japan with cells from a patient with SCC of the tongue that metastasized to a cervical lymph node. The OSC-19-luc cells had been retrovirally infected previously with the luciferase gene to allow bioluminescence imaging (BLI). Cells were grown in vitro in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, l-glutamine, sodium pyruvate, nonessential amino acids, and a vitamin solution (Life Technologies Inc, Grand Island, New York).

CELL ASSAYS

To test the sensitivity of in vitro detection of OSC-19-luc cells, 2 assays using ProSense680 and MMPSense680 were per-
formed. An increasing amount of cells (range, 0-40,000) was seeded in two 96-well plates. After 7 hours, 100 µL of ProSense680 (45 nM) was added per well in the first plate, which incubated on the cells for 24 hours. Medium without probe was added to other cells in the plate as a control. In addition, the cells were washed 3 times with a phosphate-buffered saline solution to discard excess nonbound agent according to previous studies.6 Finally, the wells were imaged using the Odyssey scanner (LI-COR Biosciences, Lincoln, Nebraska) (focus offset, 3 mm; 700-nm channel intensity, 10). The same protocol was used for the assay of MMPSense680 in the second plate.

ANIMAL MODEL

BALB/c nu/nu female mice, aged 4 to 6 weeks (Charles River Laboratories, L’Arbresle, France), were obtained and housed in accordance with the guidelines of the Animal Welfare Committee of Leiden University Medical Center, which also approved the study. All experiments were conducted and animals humanely killed according to these guidelines. The animals were housed in the animal facility of Leiden University Medical Center. Autoclaved pellet food and sterilized water were provided ad libitum. The weight of the animals was followed throughout the experiment to monitor their general health. Throughout tumor inoculation and the imaging procedures, the animals were anesthetized with 4% isoflurane for induction and with 2% isoflurane for maintenance in oxygen with a flow of 0.8 L/min and were placed on an animal bed with an integrated nose mask.

STUDY DESIGN

To induce an OSCC model, $6 \times 10^4$ OSCC-19-luc cells diluted in 30 µL phosphate-buffered saline solution were injected submucosally into the distal end of the tongue of 10 BALB/c nu/nu mice. Tumor growth was assessed 2 times a week by BLI and visual inspection of the tongue.

On day 21, mice were randomly allocated to administration of 1 of the 2 activatable NIRF agents: ProSense680 or MMPSense680. For both agents, a control group (each containing 5 BALB/c nu/nu mice without tumor) was treated with an injection of physiologic saline in the tongue and later was intravenously injected with either of the 2 activatable agents. The agents were injected intravenously (1.33 nmol, 100 µL per animal) into 1 of the tail veins. Fluorescence imaging (FLI) was performed 24 hours after injection based on the blood pharmacokinetics of the agents (VisEn Web site: http://www.visenmedical.com), and the animals were humanely killed afterward. Subsequently, the overlying skin of the cervical region was removed for gross examination. After whole-animal FLI, the tongue and cervical lymph nodes were completely excised for additional ex vivo fluorescence measurements.

BLI AND FLI

Currently, BLI is considered the most sensitive imaging technology available. A recent study has shown the ability to pick up a single cancer cell in vivo using the same imaging systems.
and luciferase vectors that were used in the present study. Because BLI requires genetic modification of the cancer cells, this technique is not applicable for clinical use, and FLI is required to identify the tumor. However, in the experimental setting, BLI can be used as an internal control to assess co-localization with the fluorescence signal.

Noninvasive BLI was performed by anesthetizing the animals with 2% isoflurane before and during imaging. An aqueous solution of luciferin (Caliper Life Sciences, Hopkinton, Massachusetts) at 150 mg/kg in a volume of 50 µL was injected intraperitoneally 5 minutes before imaging, after which the animals underwent imaging using the IVIS 100 imaging system (Caliper Life Sciences). Quantification of the BLI signal was performed through standardized regions of interest using Living Image software (version 3.2; Caliper Life Sciences). The FLI of the mouse was performed using the Maestro (CRI, Woburn) fitted with the yellow filter with an acquisition range of 630 to 800 nm in 10-nm steps and variable acquisition time.

**HISTOLOGIC ANALYSIS**

After FLI, tongues were surgically removed and snap frozen on dry ice. Subsequently, they were cut into slices (20 µm) and were imaged using the Odyssey scanner, followed by imaging using the EVOS fluorescence microscope fitted with a Cy5.5 filter (Advanced Microscopy Group, Bothell, Washington). Then, tissue sections were air-dried and stained with standard hematoxylin-eosin.

Cervical lymph nodes were surgically removed and cut into 2 halves. One half was fixed in 3.7% formalin overnight and paraffin embedded, cut into slices (20 µm), imaged using the Odyssey scanner, and hematoxylin-eosin stained. The second half was snap frozen to allow for immunohistochemical analysis. Immunohistochemical staining was performed on 10-µm-thick fresh frozen tissue sections. Rabbit polyclonal antibodies against wide spectrum cytokeratin, diluted 1:100, were used (ab9377; Abcam, Cambridge, Massachusetts). The slides were then washed with phosphate-buffered saline and detected with biotinylated polyclonal goat anti-rabbit immunoglobulins, diluted at 1:600 (E0432; Dako, Heverlee, Belgium). Slides were counterstained with hematoxylin.

After merging of the FLI with the microscopic image, co-localization of fluorescence signal with cancer tissue was determined and the TBR was calculated. To quantify the fluorescence signal, regions of interest were drawn in the tumor region and in the surrounding normal tissue at a range of 2 mm from the invasive tumor border as described previously. This was repeated in 3 different slices of the same tissue specimen, resulting in a mean TBR for each specimen, which was subsequently used for calculation of the overall TBR and standard deviation.

**STATISTICAL ANALYSIS**

Mean fluorescence intensity and associated standard deviations were assessed using the Living Image software (version 3.2) for BLI data and the Maestro software (version 2.10.0) for FLI data. Correlation was calculated using the Pearson correlation test for linear correlations and the Spearman correlation test for non-parametric data. Unpaired or paired t tests were used for testing differences in fluorescence intensity between groups. Statistical tests were 2-tailed, and \( P < .05 \) was considered significant. For statistical analysis, SPSS, version 16.0 for Windows (SPSS Inc, Chicago, Illinois), was used. GraphPad Prism software (version 5.01, GraphPad Software Inc, La Jolla, California) was used for generation of graphs, and merging of FLIs with microscopic images was performed using the Cytrion Visualization Platform (version 1.3; Leiden University).

**RESULTS**

**IN VITRO**

The fluorescence intensity of ProSense680 was significantly correlated with the number of OSC-19-luc cells...
(correlation coefficient, 0.97; P < .01) (Figure 1). This finding suggests that ProSense680 is activated by OSC-19-luc cells, indicating its potential use for in vivo testing. No correlation was observed using MMPSense680, most likely because activity of MMPs is mainly increased in the surrounding tissue of cancer cells11,12 and, therefore, is not detectable in cell culture.

IN VIVO

Seven days after inoculation of the OSC-19-luc cells, tongue tumors had developed in all 10 mice, which could be followed by BLI signal (Figure 2A). After 11 days, BLI signal indicated unilateral (n=1) or bilateral (n=9) cervical lymph node metastases in all the animals (Figure 2B). Tumor growth as a function of time (including the standard deviation) is illustrated in Figure 2C. No BLI signal was found in control animals. When FLI was performed after resection of the overlying skin in control animals, a low-level background signal of both agents was observed in the tongue and cervical lymph nodes (Figure 3, A and D). In animals with OSCC, cervical lymph node metastases were not distinguishable by gross examination after resection of the overlying skin. However, the FLIs showed clear demarcation of tumor regions in the tongue tumor and cervical lymph node metastases (Figure 3, B and E). Furthermore, quantitative comparison between tumor and control animals (n=5 per group) demonstrated significantly higher signals in malignant tissues for ProSense680 (tongue: P=.02; lymph nodes: P=.001) and MMPSense680 (tongue: P=.02; lymph nodes: P=.01) (Figure 3, G and H).

HISTOLOGIC ANALYSIS

Histologic analysis of the tissues by a pathologist confirmed the presence of OSCC in the tongue specimens of all 10 mice that had been inoculated with OSC-19-
luc cells (Figure 4 and Figure 5). Furthermore, SCC metastases were found in lymph nodes that had increased BLI signal (Figure 6). No tumor was found on hematoxylin-eosin or immunohistochemical staining analysis in lymph nodes without BLI signal.

High fluorescence signal of ProSense680 was found in the invasive border of the tongue tumor (Figure 4, B and C), which is the region where cathepsin activity is increased to facilitate degradation of the ECM. As with cathepsins, MMPs are mainly active in the direct surrounding tissue of the tumor, corresponding with the region where the fluorescence signal of MMPSense680 was found (Figure 5, B and C).

The TBR of ProSense680 in the tongue was 15.8 (SD=8.1) and in the lymph nodes was 11.8 (SD=3.6). For MMPSense680, the TBR in the tongue was 18.6 (SD=9.4) and in the lymph nodes was 10.5 (SD=4.0) (Figure 7). Subsequent imaging with brightfield microscopy with fluorescence overlay confirmed these findings (Figure 8).

In this study, OSCC with cervical lymph node metastasis was established in all the animals. Using optical imaging techniques to image the 2 activatable NIRF agents, ProSense680 and MMPSense680, we detected tongue tumor and cervical lymph node metastases. Histologic analysis demonstrated that proteolytic activity by cathepsins was highest at the invasive tumor border. This is explained by the upregulation of cathepsin B in lysosomes of tumor cells located at the invasive tumor border to degrade the ECM.9,10 These findings are in accordance with those of a study by Mieog et al,17 who demonstrated the use of ProSense680 for surgical removal of mammary cancer in a rat model. They demonstrated complete removal of the tumor under direct, real-time NIRF guidance using an intraoperative optical imaging camera system. On histologic analysis, MMP activity was mainly increased at the invasive tumor border and tumor surrounding host stromal cells, which is a well-known feature of epithelial cancers.11,12

In cancer surgery, detection of involved margins is essential for complete removal of the tumor. Therefore, it might be more important to show the border of the tumor, including the involved surrounding matrix, than the tumor mass itself. A high fluorescence signal of the tumor mass itself could potentially outshine the tumor resection margin, thereby prohibiting accurate determination of the tumor border.

A second interesting feature of MMPs is that increased activity can be used as a predictor of progression from oral dysplasia to cancer.18 In this way, NIRF imaging using MMPSense680 could potentially be used for tumor screening, follow-up of premalignant lesions, or tumor detection, which would be of great value for the current clinical practice.

It is generally accepted that a TBR greater than 2 is sufficient to critically distinguish the fluorescence signal from the background during surgery.3 In the present study, TBR was assessed on histologic samples within 2 mm of the invasive border. Values ranged from 15.8 to 18.6 for tongue tissue and from 10.5 to 11.8 for lymph nodes. This strongly indicates the feasibility of the use of both agents for real-time image-guided surgery. Based on the present results, it is expected that these agents could be used for the detection of cervical lymph node metastases in patients.

The ultimate goal of image-guided head and neck cancer surgery is complete removal of the tumor. However, further research is required to determine whether this technique will actually lead to more radical resections in clinical practice. Furthermore, before clinical studies can be performed, approval by the Food and Drug Administration to use these agents in patients is required.
In conclusion, oral cancer and cervical lymph node metastases can be detected by targeting increased proteolytic activity at the tumor borders using NIRF optical imaging. This strategy could be used for real-time image-guided surgery, which can improve the complete surgical resection rate in patients with oral cancer.

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Author Contributions: Dr Keereweer had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Keereweer, Mieog, Baatenburg de Jong, Vahrmeijer, Kerrebijn, and Lowik. Acquisition of data: Keereweer, Mol, Van Driel, and Snoeks. Analysis and interpretation of data: Mieog, Mol, Van Driel, Snoeks, and Vahrmeijer. Drafting of the manuscript: Keereweer, Kerrebijn, and Lowik. Critical revision of the manuscript for important intellectual content: Mieog, Mol, Snoeks, Baatenburg de Jong, and Vahrmeijer. Statistical analysis: Keereweer and Mieog. Administrative, technical, and material support: Mol, Baatenburg de Jong, and Lowik. Study supervision: Baatenburg de Jong, Vahrmeijer, Kerrebijn, and Lowik.

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Additional Contributions: P. C. W. Hogendoorn, PhD (Department of Pathology, Leiden University Medical Center), analyzed the tissue sections; J. N. Myers, MD, PhD (University of Texas M. D. Anderson Cancer Center, Houston), provided the OSC-19-luc cells; J. Dijkstra, Ing, PhD (Department of Radiology and Image Processing, Leiden University Medical Center), contributed to the overlay of histologic images with FLIs; and I. Que, BA (Animal Experimentation Laboratory, Leiden University Medical Center), assisted with the animal experiments.

REFERENCES