Sensitivity and Specificity of Fluorescent Immunoguided Neoplasm Detection in Head and Neck Cancer Xenografts

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Objectives: To determine whether fluorescently labeled anti–epidermal growth factor (EGFR) antibody could be used to detect residual disease and to guide surgical resections by comparing the sensitivity and specificity of optical fluorescence imaging with the sensitivity and specificity of histopathologic evaluation.

Design: A preclinical model of head and neck squamous cell carcinoma.

Subjects: Mice xenografted with SCC-1 tumor cells.

Interventions: The mice underwent systemic injection with anti-EGFR antibody (cetuximab) conjugated to an optically active fluorophore (Cy5.5). Both a subcutaneous flank model (n=18) and an orthotopic murine model (n=15) were used to assess for the presence of residual disease by fluorescent stereomicroscopy after subtotal resections of tumors. Histologic analysis was performed to confirm the presence or absence of disease.

Results: In the subcutaneous flank model, a diagnostic dose (50 µg) and therapeutic dose (250 µg) of fluorescent-labeled anti-EGFR were administered. When a diagnostic dose was given, the sensitivity was 86%, which was less than the 91% sensitivity when the higher dose was given. Tumor biopsy specimens in which disease was detected by histologic analysis but not by fluorescence (false-negative result) averaged 166 cells (range, 50-350 cells). The specificity of optical fluorescence to predict the presence of tumor in both groups was 100%. In the floor of the mouth model, we demonstrated a sensitivity of 81% and a specificity of 100%. False-negative results were obtained in a tumor fragment measuring less than 0.5 mm in diameter.

Conclusion: These data support further investigation of fluorescently labeled anti-EGFR antibody to detect disease in the surgical setting.

Arch Otolaryngol Head Neck Surg. 2007;133:511-515

Surgeons determine tumor margins intraoperatively by gross palpation, a technique that has not changed in the past 50 years. Unfortunately, this technique results in positive or close surgical margins in almost 40% of cases.¹,² Because positive margins predict poor survival, accurate identification of intraoperative margins may be of significant value in guiding the surgeon during ablative procedures. Identification of a cancer-specific optical contrast agent that could provide surgeons with real-time information about the tumor margins or the presence of residual disease has the potential to improve outcomes and to minimize sacrifice of uninvolved tissues.

Fluorescent compounds (fluorophores) absorb light at one wavelength and emit light at another wavelength. Fluorescent imaging has revolutionized molecular biology and is widely applied in small animal models of cancer to accurately detect or monitor tumor growth.³ Fluorescent microscopy has been successfully used for sentinel lymph node mapping in the operating room.⁴,⁵ Although fluorophore emissions have insufficient tissue penetration for whole-body imaging, optical fluorescence has significant potential in the surgical setting, where tissue planes are often exposed. The clinical utility of a fluorescent contrast agent will depend on the sensitivity, specificity, and toxicity of the cancer-specific probe.

Epidermal growth factor receptor (EGFR) is an excellent target for a cancer-specific contrast agent in head and neck squamous cell cancer (HNSCC) because it is highly overexpressed and is upregulated early in tumor progression.⁶,⁷ Cetuximab (Erbitux, or C225; ImClone Systems Inc, New York, NY) is a monoclonal antibody directed against EGFR.⁸ Cetuximab selectively binds to the external domain for the EGFR with high affinity (Kᵦ=1

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nmol/L)\textsuperscript{9} and has been effective in treating HNSCC when used alone or in combination with radiotherapy.\textsuperscript{10,11}

We have previously demonstrated that fluorescently labeled cetuximab, but not fluorescently labeled nonspecific human IgG, can be used to image HNSCC xenografts in vivo.\textsuperscript{12} In the current study, we hypothesize that fluorescently labeled cetuximab can detect the presence of residual tumor fragments after near-total surgical resection. To this end, we assess the sensitivity and specificity of optical fluorescence after systemic injection of fluorescently labeled cetuximab compared with histologic evaluation to detect microscopic disease in vivo.

**METHODS**

**REAGENTS**

We used cetuximab, a recombinant, human/mouse chimeric monoclonal antibody that binds specifically to the extracellular domain of the human EGFR. Cetuximab is composed of the Fv regions of a murine anti-EGFR antibody with human IgG1 heavy- and \( \kappa \)-light-chain constant regions and has an approximate molecular weight of 152 kDa. Cy5.5 (CyDye deoxynucleotides; GE Healthcare, Piscataway, NJ) was used as the far-red fluorescent marker. Cy5.5 has a broad absorption peak, with its maximum at 683 nm. Its emission maximum when coupled to IgG is at 707 nm, with a relative quantum yield of 0.28. Cetuximab was incubated with Cy5.5 reactive dye (CyDye deoxynucleotides; Amersham Biosciences, Piscataway, NJ) in 0.15 mol/L phosphate buffer at a pH of 7.8 for 1.5 hours. The nonconjugated Cy5.5 was removed using a commercially available centrifugal filter unit (Centricon Centrifugal Filter Unit YM-30; Millipore; Billerica, Mass). All procedures were performed under aseptic conditions.

**ANIMAL MODELS**

Severe combined immunodeficiency disorder (SCID) male mice (age range, 4–6 weeks) (Charles River Laboratories, Wilmington, Mass) were obtained and housed in accordance with the Institutional Animal Care and Use Committee guidelines of the University of Alabama at Birmingham, and all experiments were conducted and the animals euthanized according to the same guidelines. The SCC-1 cells were maintained in Dulbecco modified Eagle medium containing 10% fetal bovine serum and supplemented with 1-glutamine, penicillin, and streptomycin. They were incubated at 37°C in 5% carbon dioxide. The SCC-1 cells (1 X 10^5) were then injected into the subcutaneous tissue on the flank of SCID mice. The mice underwent systemic tail vein administration of the conjugated antibody before optical imaging. Three days after cetuximab-Cy5.5 injection, subcutaneous flank or transversal surgical resections were performed on euthanized mice. Stereomicroscopic fluorescent imaging was performed before and after each near-total surgical resection.

To determine the sensitivity and specificity of fluorescently labeled cetuximab to detect HNSCC xenografts, we used 2 separate models for analysis. In the subcutaneous flank model, tumors were measured biweekly with calipers until they reached a maximal diameter of 5 to 10 mm (usually 2-3 weeks’ incubation time) and then injected with either a diagnostic dose (50 µg; n = 8 mice) or a therapeutic dose (250 µg; n = 8 mice) of cetuximab-Cy5.5 three days before imaging. We classified doses in this manner to provide the reader with a clinical context: what we classify as the therapeutic dose has been shown in previous studies to slow progression of tumors in xenograft models,\textsuperscript{13} whereas the diagnostic dose represents a subtherapeutic dose with fewer potential toxic effects. At 72 hours after injection of the cetuximab-Cy5.5 conjugate, subtotal resection was performed and microcup biopsy forceps (Hartmann-Herfeld Cup-Shaped Alligator Forceps, 2 mm cup size; Medtronic, Jacksonville, Fla) were used to sample the wound bed during fluorescent imaging. Biopsy specimens were obtained from fluorescent and nonfluorescent areas of the wound bed after near-total tumor resection and processed for histopathologic examination. Because the biopsy forceps were small (2-mm) and the wound beds larger than 1 cm, usually 2 fluorescent-positive and 2 fluorescent-negative biopsy specimens could be obtained from each wound bed. The hematoyxilin–eosin–stained sections from false-negative 2-mm biopsy specimens were reviewed to determine the number of tumor cells present in the samples. The number of cells was counted by examination of serial sections (“bread loaf”) of the biopsy specimens sectioned at 100-µm intervals under magnification. In the orthotopic model, the mandible and associated structures were removed and paraffin embedded after surgical resections. Serial (bread loaf) histologic sectioning and routine hematoyxilin–eosin staining were then performed to assess the presence and location of residual tumor. A total of 15 SCID mice were injected in the floor of the mouth transcervically (4 X 10^5 cells). The animals were anesthetized biweekly, and the floor of the mouth and the neck were palpated for the presence of tumor. Once palpable tumors were detected (4-8 mm), the mice underwent systemic administration of cetuximab-Cy5.5 (50 µg) 3 days before imaging. Each half of the mandible (left and right) was evaluated by stereomicroscopic fluorescence, the findings of which were then correlated with histologic findings of the serially sectioned mandibles. Therefore, each mouse (with 2 mandible halves for evaluation) was counted as 2 data points, and because 15 mice were included in the study, the total was 30 data points.

**IMAGING**

Human tumor xenografts were imaged using a custom-built fluorescent stereomicroscope (Leica MZFL3 Stereo Research Microscope; Leica Microsystems, Bannockburn, Ill), which was fitted with a Cy5.5 filter, and a high-resolution digital charge-coupled device camera (ORCA ER; Hamamatsu, Bridgewater, NJ) to allow real-time imaging of Cy5.5 fluorescence. A Cy5.5 filter (Chroma filter set 41023; Chroma Technology, Rockingham, Vt) provided excitation between 630 and 670 nm, and emission was measured at 685 to 735 nm. Gross, brightfield, and fluorescent images were obtained for each resection.

**STATISTICAL ANALYSIS**

Sensitivity, specificity, positive predictive value, negative predictive value, and confidence intervals were calculated for each comparison using the normal approximation of the standard error for proportions. The confidence intervals were then calculated using the t test for normally distributed data at the specified confidence level.

**RESULTS**

**SUBCUTANEOUS FLANK XENOGRAFT MODEL TO ASSESS CETUXIMAB-CY5.5’s SENSITIVITY AND SPECIFICITY**

The SCC-1 tumor cell line was used to generate subcutaneous flank tumors in 18 mice. Subtotal resections were performed, and the wound was examined by fluorescent stereomicroscopy. To validate fluorescent findings, multiple biopsy specimens from the wound bed were
directed at both fluorescent and nonfluorescent areas using a 2-mm cup forceps (Figure 1) and processed for histologic analysis. A diagnostic dose (50 µg; n=8 mice) or a therapeutic dose (250 µg; n=8 mice) was administered in this model. When a diagnostic dose was administered, fluorescence predicted the presence of tumor in 16 of 16 biopsy specimens, a sensitivity of 100% (Table 1). When areas of negative fluorescence were sampled from the wound bed after near-total resection, fluorescence failed to predict the presence of disease in 3 of 22 biopsy specimens, a sensitivity of 86%. Serial sectioning of the biopsy specimens was performed, and the number of cells in these false-negative specimens was approximated. We identified an average of 166 cells per false-negative specimen (range, 50-350 cells). Representative true-positive and false-negative biopsy specimens are shown in Figure 2. In the group that received a therapeutic dose of cetuximab-Cy5.5, fluorescence predicted the presence of cancer in 23 of 23 cases, a sensitivity of 100%. When biopsy specimens were obtained from areas that demonstrated no fluorescence on imaging within the wound bed (an example of a negative fluorescent wound site is indicated by the gray arrow in Figure 1D), the presence of disease was detected in 1 of 11 samples (50 cells were identified in this biopsy sample).

**FLOOR-OF-MOUTH MODEL TO ASSESS CETUXIMAB-CY5.5’s SENSITIVITY AND SPECIFICITY**

A total of 15 mice were inoculated with the SCC-1 tumor cell line transcervically in the floor of the mouth and then imaged after subtotal resection. The left and right halves of the mandibles were then analyzed separately, for a total of 30 data points (Figure 3). The presence of fluorescence was confirmed by the presence of tumor in 14 of 14 samples, for a specificity of 100% (Table 2). Fluorescent imaging failed to detect tumor in 3 of 16 samples in which there was histologic evidence of tumor, a sensitivity of 81%. The tumor fragments that could not be detected by fluorescence were less than 0.5 mm in diameter.

**COMMENT**

Surgeons currently rely on subtle tissue changes and frozen section for intraoperative assessment of tumor margins. Our experiments were designed to demonstrate the potential utility of fluorescently labeled cetuximab to guide surgical resections or to direct frozen section sampling intraoperatively. We demonstrate that fluorescent immunoguided neoplasm detection (FIND) using fluorescently labeled cetuximab has 100% sensitivity and 81% to 91% specificity for detecting residual disease after subtotal resection of xenografted HNSCCs. Our results suggest that the accurate detection of tumor using FIND is limited to the detection of tumor fragments of greater than 500 cells or approximately 0.5 mm in diameter.

The concept of using fluorescently targeted agents has been largely neglected by the imaging community because fluorescent probes have very limited tissue penetration. However, in the surgical setting, where the underlying tissues are usually exposed, the real-time capabilities of optical imaging may be useful to the clinician. Other rapidly developing imaging technologies, such as positron emission tomography, cannot provide clinicians with real-time information. In a separate study, we demonstrated that fluorescently labeled cetuximab is selectively taken up by head and neck cancer xenografts in an orthotopic mouse model. In that study, we also demonstrated that fluorescently labeled nonspecific human IgG does not localize to the tumor after 6 hours. This technique has not yet been evaluated as a tool to guide surgical resections. Cetuximab labeled with a fluorophore has been assessed after topical application of conjugate premalignant mucosal epithelium and normal tissue in 500-µm-thick multilayer tissue constructs. Near-infrared optical imaging has been applied to the detection of xenografted tumors using fluorescently labeled antibodies with success in Kaposi sarcoma and breast cancer. However, to our knowledge, the sensitivity and specificity of this technique in mock tumor resections have not been reported for any tumor type.

**Table 1. Flank Xenograft Model Results**

<table>
<thead>
<tr>
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<th>Diagnostic Dose, 50 µg</th>
<th>Therapeutic Dose, 250 µg</th>
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<tbody>
<tr>
<td>Sensitivity</td>
<td>86 (72-100)</td>
<td>91 (74-100)</td>
</tr>
<tr>
<td>Specificity</td>
<td>100 (100-100)</td>
<td>100 (100-100)</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>100 (100-100)</td>
<td>100 (100-100)</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>84 (68-100)</td>
<td>96 (88-100)</td>
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Abbreviation: CI, confidence interval.
Although the concept of fluorescent imaging to guide surgical therapy is not novel, a cancer-specific optical contrast agent has not been identified. Fluorescent optical imaging has been performed in neurosurgery to guide surgical resections. Surgeons have used indocyanine green in both abdominal and thoracic surgical procedures to identify sentinel lymph nodes. Although optical imaging technology can be readily adapted to the operating room, it has not been widely used because currently approved fluorophores lack tumor specificity. Indocyanine green is approved for use in humans but cannot be easily linked to proteins. In response to demands from molecular biologists, novel fluorophores have been generated that can be linked to antibodies without disrupting the ligand-binding site. Significantly, the development of therapeutic antibodies that selectively bind to tumor cells allows tumor-specific delivery of the fluorophore.

The findings of histologic analysis of the tumor fragments that could not be detected by fluorescence suggest that tumor fragments smaller than 0.5 mm in diameter or composed of fewer than 500 cells cannot be detected with FIND. A higher dose of fluorescently labeled cetuximab did improve the sensitivity of this technique from...
88% to 91%. It is likely that some parts of the tumor either do not bind the fluorescently labeled probe (lack of EGFRs) or are not exposed to it (reduced blood flow or partial necrosis). It is possible that the use of different fluorophore or targeting agents may improve the limits of detection.

In the current study, 2 preclinical models of HNSCC with different tumor growth were used to assess the utility of FIND and yielded similar results. The sensitivity was 100% in both the subcutaneous flask and the orthotopic models, suggesting that fluorescence that was detected within the wound bed could be considered tumor. However, we identified a range of sensitivity between 81% and 91%, depending on the model, suggesting that negative fluorescence could not be consistently identified as tumor.

In conclusion, our study findings provide evidence that fluorescently labeled anti-EGFR antibody has excellent sensitivity and good specificity. The FIND detection technique with the cetuximab-Cy5.5 conjugate appears to be limited to tumor fragments measuring approximately 0.5 mm in diameter. These data support the further investigation of fluorescently labeled cetuximab as a cancer-specific contrast agent to guide surgical therapy or to detect disease in the clinical setting.

Submitted for Publication: August 1, 2006; final revision received October 17, 2006; accepted December 3, 2006.

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Author Contributions: Dr Rosenthal 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: Magnuson, Zinn, and Rosenthal. Acquisition of data: Kulbersh, Duncan, and Skipper. Analysis and interpretation of data: Kulbersh, Magnuson, Zinn, and Rosenthal. Drafting of the manuscript: Kulbersh and Rosenthal. Critical revision of the manuscript for important intellectual content: Magnuson, Skipper, and Zinn. Statistical analysis: Kulbersh and Rosenthal. Obtained funding: Zinn and Rosenthal. Administrative, technical, and material support: Kulbersh and Skipper. Study supervision: Zinn and Rosenthal.

### Table 2. Floor-of-Mouth Orthotopic Results

<table>
<thead>
<tr>
<th>Results</th>
<th>% (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>81.250 (62.125-100.000)</td>
</tr>
<tr>
<td>Specificity</td>
<td>100.000 (100.000-100.000)</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>100.000 (100.000-100.000)</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>82.353 (64.231-100.000)</td>
</tr>
</tbody>
</table>

Abbreviation: CI, confidence interval.

### Financial Disclosure

None reported.

### Funding/Support

This work was supported by grant RSG-06-1006-01-CCE from the American Cancer Society and by University of Alabama at Birmingham Comprehensive Cancer Center Core Grant NIH P30 CA13148.

### Previous Presentation

This study was presented at the American Head and Neck Society 2006 Annual Meeting and Research Workshop on the Biology, Prevention, and Treatment of Head and Neck Cancer; August 19, 2006; Chicago, Ill.

### Acknowledgment

The SCC-1 cell lines were a gift from Thomas Carey, PhD, University of Michigan, Ann Arbor.

### REFERENCES