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Fluorescently-Labeled Cetuximab-IRDye800 for Guided Surgical Excision of Ameloblastoma: A Proof of Principle Study

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Abstract

Purpose: Fluorescently labeled epidermal growth factor receptor (EGFR) antibodies can successfully identify microscopic tumors in multiple in vivo models of human cancers with limited toxicity. This study demonstrates the ability of fluorescently labeled anti-EGFR, cetuximab-IRDye800, to localize to ameloblastoma tumor cells in vitro and in vivo.

Material and Methods: EGFR expression in ameloblastoma cells was confirmed by qRT-PCR and immunohistochemistry. Primary ameloblastoma cells were labeled in vitro with cetuximab-IRDye800 or non-specific IgG-IRDye800. An in vivo patient-derived xenograft model of ameloblastoma was developed: tumor tissue from three patients was implanted subcutaneously into immunocompromised mice, animals received intravenous injection of cetuximab-IRDye800 or IgG-IRDye800, and were imaged to detect infrared fluorescence using a LI-COR Pearl imaging

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system. Following overlying skin resection, tumor to background ratios (TBR) were calculated and statistically analyzed by paired t-test.

Results: EGFR expression was seen in all ameloblastoma samples. Tumor-specific labeling was achieved, as evidenced by positive fluorescence signal from cetuximab-IRDye800 binding to ameloblastoma cells, with little staining seen in the negative controls treated with IgG-IRDye800. In the animal PDX model, imaging revealed the tumor-to-background ratios (TBRs) produced by cetuximab were significantly higher than those produced by IgG on days 7–14 for AB-20 tumors. Following skin flap removal to simulate a pre-resection state, TBRs increased with cetuximab and were significantly higher than the IgG control for PDX tumors derived from three ameloblastoma patients. Excised tissues were paraffin-embedded to confirm the presence of tumor.

Conclusions: Fluorescently labeled anti-EGFR demonstrates specificity for ameloblastoma cells and PDX tumors. This study is the first report of tumor-specific, antibody-based imaging of odontogenic tumors, of which ameloblastoma is one of the most clinically aggressive. We expect this technology will ultimately assist surgeons treating ameloblastomas by helping them to accurately assess tumor margins during surgery, leading to improved long-term local tumor control and less surgical morbidity.

Keywords

ameloblastoma; receptor; epidermal growth factor; cetuximab; optical imaging; animals; head and neck neoplasms

Introduction:

Ameloblastomas, one of the most common odontogenic neoplasms, are known for locally aggressive and destructive behavior, with approximately 96% occurring as intraosseous tumors primarily within the posterior mandible.^{1,2} Histologically, ameloblastomas are similar to basal cell carcinomas, sharing many features of low-grade malignancy. Untreated, they steadily destroy the jaws, paranasal sinuses, and nasal cavity and invade vital structures such as the skull base or dura, and have been known to metastasize to lungs, long bones, and cervical lymphatics.^{3,4} Despite their destructive phenotype, there is no international consensus among clinicians regarding appropriate treatment. Some advocate for a subtotal excision consisting of enucleation and curettage, sparing nerve, bone, and teeth, while others perform segmental resection.^{5–11} As a result of the surgeon's inability to visualize tumor margins intraoperatively, as well as the varying opinions regarding treatment, ameloblastoma recurrence ranges from 6–52% after surgery.^{5–10} Ameloblastomas also tend to recur many years after conservative treatment, ostensibly due to the lack of margin control with these procedures.^{11–14}

Currently, intraoperative tumor evaluation methods are lacking, especially in intraosseous tumors, resulting in positive margins in approximately 30% of head and neck cancer resections.¹⁵ During surgery, tumor margins are typically assessed grossly by visual inspection and palpation. In the case of intrabony tumors like ameloblastoma, radiographs of the resected specimen may help identify the radiolucent tumor edges, but does not allow for microscopic or tumor-specific margin assessment.¹⁶ Guided by nonspecific methods,

ameloblastoma tumor resection surgery has not changed in many decades. Frozen section histology is useful for evaluating soft tissue adjacent to intrabony tumors for confirmation of diagnosis;¹⁷ however, is not practical for bone margin assessment due to the need for decalcification and sectioning, which requires up to 14 days. Intraoperative fluorescent tumor visualization is now possible using precise antibody-specific navigation in a number of human tumors (Table 1).^{18–55} The ability of the surgeon to see gross tumor fluoresce under NIR imaging in the operating room, and identify microscopic tumor deposits in the frozen section room, reduces the chance for positive margins and increases long term tumor control.⁵⁶ Applying antibody-based, tumor specific technology to ameloblastoma treatment may reduce recurrence of ameloblastoma during conservative ablative surgery while also preserving normal bone, teeth, and soft tissue.

Several FDA-approved anti-neoplastic antibodies can be modified for use in fluorescence-guided surgery, including anti-EGFR drugs like cetuximab and panitumumab, by adding a fluorophore to the Fc region of the antibody. When excited with light at a particular wavelength the fluorophore, antibody, and attached tumor cell fluoresce and can be detected both grossly and microscopically. Similar to therapeutic drug delivery for head and neck cancers, the antibody + optical probe is delivered via intravenous infusion, however is dosed sub-therapeutically to minimize toxicity and ensure safety. Antibody-based optical probes are being adopted widely in a number of tumor types, but have not been reported in ameloblastoma treatment.^{18,26,31,32,42}

The specific aim of this study was to determine if ameloblastoma, which expresses EGFR on its cell surface, could be safely excised in a pre-clinical setting using fluorescent antibody-based surgical navigation.^{57–64} To complete this study we created a novel ameloblastoma tumor model both in vitro and in vivo, then used a unique fluorescent contrast probe (cetuximab IRDye-800) to visualize the tumors both histologically and macroscopically. Initially, we hypothesized that EGFR, expressed on the surface of ameloblastoma cells, could be exploited to identify and label tumor cells in vivo^{57–64}. This was carried out using cell culture and qRT-PCR of biopsy-confirmed ameloblastoma cells and immunohistochemistry. Next, to demonstrate the ability of the antibody-based probe cetuximab-IRDye800 to detect ameloblastoma, we developed a novel patient-derived xenograft (PDX) model from multiple ameloblastoma patients undergoing surgery, then imaged mice using closed and open-field techniques to mimic the clinical scenario. This is the first study to use this new mouse model for fluorescent ameloblastoma tumor imaging, and the first study demonstrating PDX of ameloblastoma.

Materials and Methods

Tumor specimens and preparation

Tissue samples were collected from patients diagnosed with ameloblastoma who underwent surgical resection at the University of Alabama at Birmingham (UAB) from 2015 to 2017. During the study period, 40 patients were identified with a diagnosis of recurrent or primary ameloblastoma requiring a microvascular free flap, indicating a larger tumor which offered adequate material for implantation. Of these, three patients both consented to the study prospectively and were felt to have sufficient tumor volume available for xenograft

preparation and implantation into mice. All patients had multicystic/ solid ameloblastoma with mandibular bone destruction while the salivary glands and lymph nodes were negative for tumor.

The study was independently reviewed and approved by the UAB Institutional Review Board and informed consent was obtained from all patients. The ablative surgeon (A.M.) obtained tumor tissue for the study distant from the specimen edges, in a manner that did not interfere with the pathologist's ability to assess surgical margins. To confirm the presence of tumor prior to implantation, a portion of tissue was analyzed by a board-certified pathologist using frozen section techniques. Once tumor presence was confirmed, the explants were dissected into 2×2 mm pieces, rinsed in DMEM/F-12 media supplemented with penicillin/streptomycin and amphotericin (ThermoFisher, Waltham, MA). The remainder of the surgical specimens were processed by the pathology department per standard of care for surgical pathology, and slides generated which were formalin-fixed and paraffin-embedded (FFPE).

Cell culture and immunofluorescence with cetuximab-IRDye800

Six patient-derived ameloblastoma (AB) cell populations were isolated from outgrowths of fresh, minced patient samples (procedure described^{65,66}). All AB cells were maintained in DMEM media supplemented with 10% FBS and antibiotics at 37°C in 5% CO₂. HNSCC cell lines were purchased from American Type Tissue Collection (Manassas, VA) and maintained per vendor instructions. For immunofluorescence, AB cells (2.5×10^4) were grown 4-well glass chamber slides and fixed with 4% formaldehyde, blocked with 10% BSA and incubated with cetuximab-IRDye800 or IgG-IRDye-800 overnight at 4°C. Cells were rinsed with phosphate buffered saline and imaged on the Odyssey imaging system at 800 nm (LI-COR Biosciences, Lincoln, NE).

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from HNSCC and ameloblastoma (AB) cell populations using the Qiagen RNeasy Mini Kit (Valencia, CA) and converted to cDNA using the Applied Biosystems High-capacity RNA-to-cDNA kit (Waltham, MA) according to manufacturer's instructions. qRT-PCR reactions were conducted with the SABiosciences SYBR Green qPCR master mix (Frederick, MD) and EGFR transcript levels were detected on the 7500 Real-Time PCR System (Applied Biosystems). EGFR primers used were Qiagen RT² qPCR assays (PPH00138B-200, Germantown, MD). GAPDH primers (hGAPDH F 5'-AGGTCGGAGTCAACGGATTTG-3'; hGAPDH R 5'-TGTAACCATGTAGTTGAGGTCA-3') were synthesized by IDT. (Cycle threshold (Ct) values were obtained and normalized to the GAPDH reference gene.

Immunohistochemistry

FFPE tissues sections (5 µm) were cut, deparaffinized, and rehydrated. Sections were in citrate antigen retrieval buffer for 20 minutes at 97°C and blocked in 3% hydrogen peroxide for 10 minutes. Samples were incubated with EGFR antibody (31G7, Invitrogen, 1:100) overnight at 4°C. Horseradish peroxidase polymer conjugated antibody was applied for 10 min and detected using diaminobenzidine tetrachloride with hematoxylin counterstain

(Invitrogen, Carlsbad, CA). Negative control was processed with secondary antibody and no primary (data not shown).

Animal model

Athymic mice (Envigo, East Millstone, New Jersey), aged 3–6 weeks, were obtained and housed according to UAB Animal Resource Program guidelines, following approval by the Institutional Animal Care and Use Committee (IACUC). Mice were anesthetized with isoflurane and incision sites prepared with alternating betadine and 70% ethanol in triplicate. To implant the PDX tumors, an incision was made through the skin of the left flank, a subcutaneous pocket developed between the skin and muscle, the implant immobilized with suture in the supramuscular plane, and the wound closed with staples. Mice were treated with carprofen prior to incision to reduce post-operative pain. The mice were imaged at 4–6 weeks following PDX implantation, allowing optimal tumor revascularization prior to injection of the fluorescent antibody probe. After establishing the animal models, we performed a tail vein injection with fluorescently labeled anti-EGFR antibody (cetuximab-IRDye800) or non-specific IgG (IgG-IRDye800) as control to determine the specificity of cetuximab-IRDye800 to ameloblastoma *in vivo*. Conjugation of antibodies to IRDye800 (LI-COR Biosciences, Lincoln, NE) was performed as previously described.^{43,55} Mice were randomly divided into groups receiving different treatments: cetuximab-versus IgG control, then received an intravenous tail vein injection of 200 µg of cetuximab-IRDye800 or IgG-IRDye800.⁴² Animals were then imaged using the near-infrared LI-COR Pearl imaging system at 0, 4, 7, 10, and 14 days post-antibody injections. At 14 days post-injection, animals were sacrificed and the PDX tumors were resected. All tumors were excised, imaged *ex vivo*, and stored for pathologic analysis.

Fluorescent imaging and measurement

The Pearl Impulse Small Animal Imager (LI-COR Biosciences, Lincoln, NE) is designed to detect near-infrared fluorescence *in vitro* and *in vivo*. Following injection with cetuximab-IRDye800 or IgG-IRDye800, animals were imaged on the Pearl Imager with a 785-nm excitation and 820-nm emission filter. To measure the tumor-to-background (TBR) ratios, the mean fluorescence intensity (MFI) was determined for each tumor by defining a region of interest (ROI) around the tumor and an adjacent non-tumor background area. ROIs were analyzed using the LI-COR Image Studio software (Version 3.1) to quantify the MFI. The TBR is calculated by normalizing the tumor MFI to the background MFI and averaging the TBRs in each tumor group.^{18,26,32,42,67} We also used the Luna open-field surgical imaging unit to confirm the usefulness of this technique in the operating room.

Statistical analysis

TBRs of the cetuximab-IRDye800 and IgG-IRDye800 groups were compared for each time point by a paired Student's t-test. Significance was considered to be $p < 0.05$. Errors bars represent standard error.

Results

Expression of EGFR in ameloblastoma tumors

EGFR expression was detected in six AB patient-derived cell populations and compared to four head and neck squamous cell carcinoma (HNSCC) cell lines, known to avidly express EGFR, via qRT-PCR.^{68–70} All the AB tumor cell populations demonstrated high expression of EGFR and at similar levels as HNSCC tumor cell lines (Fig 1A). We expected EGFR expression in our cell lines to mirror reported expression of EGFR in the current literature.^{57,59–63}

Demonstrating Tumor-specific Fluorescence in Vitro

To demonstrate the ability of cetuximab-IRDye800 to bind to AB cells, we labeled AB cells with cetuximab-IRDye800 (“cetux”, green fluorescence). As a control, AB cells labeled with the negative control IgG-IRDye800 (IgG) showed little to no staining with a lack of green fluorescence (Fig 1B).

Next, the protein expression of EGFR was confirmed in formalin-fixed paraffin embedded tissue sections of AB from 10 patients (Fig 1C, 4 pictured). This battery of experiments demonstrates the ability of cetuximab-IRDye-800 to bind AB cells preferentially over IgG-IRDye800.

Detection of cetuximab-IRDye800 in vivo

To generate the PDX model, we implanted surgical samples of ameloblastoma into athymic nude mice. Tumor xenografts from one patient with an especially large tumor (AB-20) were implanted into 12 mice. The resulting xenografts were imaged *in vivo* on days 0, 4, 7, 10, and 14 following tail vein injection of 200 µg of cetuximab-IRDye800 or IgG-IRDye800. The tumor-to-background ratios (TBRs) produced by cetuximab were significantly higher than those produced by IgG by day 7 ($p=0.04$) and remained significantly higher on days 10 and 14 ($p=0.01$ and $p=0.006$, respectively) (Fig 2). On day 14, the cetuximab-IRDye800 TBRs ranged from 2.35–4.12 ($n=8$, mean=2.95) and the IgG-IRDye800 TBRs ranged from 1.34–2.40 ($n=4$, mean=1.86). Representative images of mice from day 14 showed higher intensity than seen in the cetuximab-IRDye800 mouse (Fig 2).

Simulating resection of ameloblastoma using fluorescence imaging guidance: Gross and Microscopic Tumor Detection

a. In Situ Gross Tumor Detection: To simulate surgical excision of ameloblastoma using the fluorescent cetuximab-IRDye800 for guidance, imaged the whole mouse, with the PDX ameloblastoma flank implant, using the LI-COR unit. We then removed the skin and hair directly over the implanted tumor in the mouse flank, and then imaged the animals again, comparing the TBR before and after skin excision. In the cetuximab-IRDye800 mice, mean TBR following skin excision was 4.44 compared to 2.95 prior to skin removal, compared to the 1.86 and 1.83 pre-and post-skin excision, respectively, for the IgG-IRDye800 animals. This example is significant because it simulates the appearance of ameloblastoma tumors in the jaw during soft tissue approach, as seen in surgery. We observed an increase in TBR for cetuximab-IRDye800 when tumor site was exposed

directly, but no change in the IgG-IRDye800 TBR. The TBRs for cetuximab-IRDye800 treated mice were significantly greater than those treated with IgG (Fig 3, $p=0.003$). Ameloblastoma tissue from two additional patients (AB-33, AB-34) were implanted in the flanks of mice. Animals again randomly received either cetuximab-IRDye800 or IgG-IRDye800. Fourteen days post-injection the skin flap over the tumor area was removed. When the skin flap was removed, the TBRs produced by cetuximab-IRDye800 for these samples were significantly higher than those produced by IgG-IRDye800 (Fig 3; AB-33, $p=0.013$; AB-34, $p=0.027$). The TBRs for AB-30 tumors ranged from 2.22–3.68 (mean=2.88) for cetuximab-IRDye800 treated animals and 1.76–2.63 (mean=2.01) for IgG-IRDye800 treated. For the AB-34 tumors, the cetuximab-IRDye800 treated TBRs were 1.63–3.01 (mean=2.1) and the IgG-IRDye800 TBRs were 1.29–1.51 (mean=1.44).

b. Specificity of Cetuximab-IRDye800 to Ameloblastoma Tumor Stroma: To demonstrate the specificity for cetuximab-IRDye800 to ameloblastoma tumor tissue, we also implanted tumor-associated stroma from an ameloblastoma patient (tissue piece was from tumor adjacent area from surgical resection). The TBRs of tumor-associated stroma from cetuximab-IRDye800 treated animals were 1.23–1.96 (mean=1.76) and IgG-IRDye800 treated animals were 1.47–1.90 (mean=1.73) with no significant difference ($p=0.836$).

c. Detecting minute fragments of Ameloblastoma for potential intraoperative margin assessment—To determine the capacity of cetuximab-IRDye800 to detect minute disease, tumor fragments (5, 1, and 0.5 mg) were placed on a wound bed of skeletonized muscle, devoid of tumor, and imaged (Fig 4). We were able to detect all pieces of tumor, including the 0.5 mg piece, showing the ability to detect even small amounts of tumor (Fig 4). In our samples, 1.3–25.0% of solid tumor explants contained viable ameloblastoma cells, while 75–98.7% was felt to consist of stroma and non-tumor tissue.

d. Ex vivo histopathologic confirmation of ameloblastoma—To validate our PDX model, tumors were resected on post-imaging day 14, paraffin-embedded and stained with hematoxylin and eosin. Microscopic examination of each explanted xenograft by a board-certified pathologist (T.S.) confirmed the presence of ameloblastoma amongst tumor-associated stroma (Fig 5).

Discussion

In the present study, we confirm the expression of EGFR in ameloblastoma tumor tissue and show ameloblastoma cells expressed similar levels of EGFR as SCC cells, consistent with a number of studies reporting the expression of EGFR in ameloblastoma tissue.^{57–64} Of 218 ameloblastoma cases reported, only 3 did not express EGFR on the tumor cell surface. In other publications, EGFR is overexpressed compared to normal oral mucosa, tumor-associated stroma, and other oral tumors such as calcifying cystic odontogenic tumor and keratocystic odontogenic tumor.^{61,63} Oikawa et al, however, reported no genetic amplification of EGFR and no significant increase in EGFR compared to dental follicle or oral cysts.⁵⁹ EGFR expression is also highly variable in HNSCC clinical samples and established cell lines⁷¹, however, this has not curtailed the use of monoclonal antibody therapies in the treatment of head and neck cancer or in fluorescent surgical navigation.⁷²

Here we report EGFR is expressed in ameloblastoma cells and tissues at a level similar to head and neck squamous cell carcinoma (HNSCC) cells, an important concept since EGFR is already used in surgical navigation for HNSCC. In our study, the anti-EGFR antibody, cetuximab-IRDye800, labeled ameloblastoma cells in vivo, while the fluorescent control antibody IgG-IRDye800 did not. In this study, the first reported model of ameloblastoma xenografts, PDX tumors were established from three separate human tumors in athymic mice. In vivo, cetuximab-IRDye800 had significantly higher TBRs compared to IgG-IRDye800. In a pre-resection model, the TBRs for cetuximab-IRDye800 increased with the removal of the overlying skin flap and were significantly higher than the IgG-IRDye800 TBRs demonstrating specificity of cetuximab-IRDye800 for ameloblastoma tumor tissue. Implanted tumor-associated stroma showed no difference in TBRs between cetuximab-IRDye800 and IgG-IRDye800, further demonstrating cetuximab-IRDye800 is specific for ameloblastoma. We also demonstrated the ability of cetuximab-IRDye800 to detect minute volumes of ameloblastoma, which is significant in ultimately translating this work to the operating theater. The size of the minute fragments of ameloblastoma were selected from previous publications, are within the detectable range of cetuximab-IRDye800, and were felt to mimic disease seen in the clinical setting.^{73,74} These findings establish cetuximab-IRDye800 as a tumor-specific, highly sensitive agent to fluorescently localize ameloblastoma tumors in vitro and in vivo.

Clinical assessment of surgical margins is very challenging for intraosseous tumors due to the need for decalcification of bone prior to assessment, which requires days to weeks after surgery. Current imaging modalities used for intraoperative detection of ameloblastoma are non-specific, and include plain film radiography, computed tomography (CT), magnetic resonance imaging (MRI), contrast-enhanced MRI, or cone beam CT.^{75,76} Each of these approaches lacks tumor specificity does not readily define the surgical margins. A clinical unmet need is to provide the surgeon with an immediately available, specific, and minimally invasive tool to aid in the detection and safe removal of ameloblastoma tumors from the native maxilla and mandible. In this manuscript, we present the first adjunctive modality for ameloblastoma surgery based on fluorescent imaging technology, which presents an opportunity for translation to the clinical setting. Here we present the use of a fluorescently conjugated antibody specific to EGFR to label ameloblastoma in vitro and in vivo. EGFR is an established therapeutic target and our ameloblastomas, as well as recent studies have shown its expression in the majority of ameloblastomas examined, including solid/multicystic (plexiform and follicular), unicystic, and desmoplastic subtypes (n = 203 cases).^{57,59–63} We were able to detect a 0.5 mg piece of tumor tissue and in samples with as little as 1.3–25% ameloblastoma cells compared to stroma tissue demonstrating the feasibility of detect minute areas of tumor appropriate for margin assessment.

EGFR is also an attractive target as two EGFR-specific antibodies have been approved by the FDA for use in humans to treat HNSCC. Cetuximab and panitumumab have been conjugated to the fluorescent probe, IRDye800, by annealing the probe to the Fc segment of the antibody. Cetuximab-IRDye800 was shown to have high specificity for human EGFR in a binding assay compared to the nonspecific fluorescent antibody IgG-IRDye800. In multiple tumor models, cetuximab-IRDye800 fluorescence was able to detect distinguish tumor from normal tissue compared to the control IgG-IRDye800.^{18,26,32,42,67} In a model of

subcutaneous squamous cell carcinoma (SCC), xenografts were imaged, then resected via conventional methods (removing gross palpable tumor with 1–2mm margins). Next, the tumor area was imaged again identifying areas of residual disease not previously detected, which were then resected as well.⁶⁷ Histologic analysis of residual pieces was positive for tumor cells indicating 100% sensitivity for the labeled anti-EGFR antibodies and demonstrating improved margin resection using real-time optical imaging compared to conventional resection using inspection and palpation. To further demonstrate the specificity of the antibodies, samples of fluorescently negative tissue within 1cm of the margin were examined and found to have no evidence of tumor cells. In another model, removal of breast cancer xenografts demonstrated specific staining and uptake within the tumor areas compared to normal tissue.¹⁸ To assess the uptake of labeled anti-EGFR antibody in human tumor tissue, grafts from a patient with SCC were transplanted into the flanks of nude mice in a human explant study.²⁶ After successful transplantation, mice were injected with labeled antibodies and tumor was imaged using both open- and closed-field fluorescence imaging systems. Upon resection, explant tissue was examined by histology and fluorescent imaging to correlate fluorescent signaling with areas with confirmed tumor cells. These studies demonstrated the utility of sub-therapeutic dosing of fluorescently labeled EGFR antibodies cetuximab and panitumumab for the real-time optical imaging and resection of human HNSCC, melanoma, and breast cancer *in vivo*. In a first-in-humans study, cetuximab-IRDye800 showed significantly higher fluorescence in HNSCC tumor tissue compared to normal tissue in patients receiving 25 and 62.5 mg/m².³¹ These doses were associated with limited toxicity (no adverse events above grade 2). Post-resection tumor fluorescence correlated with tumor EGFR expression.

A limitation of this study is use of the LI-COR Pearl imaging system, which is ideal for detecting the IRDye800 fluorescent signaling, but only capable of imaging small specimens and animals. This may be useful post-operatively to examine the margins of resected specimens in surgical pathology; however, would not be useful for real-time intraoperative surgical navigation. For improved clinical application, follow-up studies are planned, incorporating open-field near-infrared imaging systems, which are currently available in the operating room and have been used for preclinical and clinical surgical navigation.^{26,31–33,42,67,77} Also, historically it is difficult to image tumors within bone, and no consensus has been reached regarding the optimal imaging strategies in ameloblastoma patients.^{78,79} Fluorescence-guided optical imaging provides the benefit of intraoperative assessment with FDA-approved imaging equipment, as well as the lack of radiation exposure experienced with PET/CT.^{33,80–82} Studies examining the use of near infrared (NIR) imaging in bone have effectively identified prostate and breast cancer cells, glioblastoma, and osteosarcoma intracranially, and in the spine and tibia of mice.^{80–82} However, the NIR agents used in the studies lack the specificity and targeting associated with cetuximab-IRDye800, which specifically binds the EGFR expressed by tumor cells. Additional studies will be needed to confirm ameloblastoma can be effectively detected intraosseously with cetuximab-IRDye800; if effective, it could improve success in surgical resections.

Our data show that the fluorescently conjugated EGFR antibody, cetuximab-IRDye800, is effective in labeling ameloblastoma tissue using a novel animal model. Fluorescently

labeling tumor tissue may be advantageous to use in conjunction with current methods to analyze tumor location and assess tumor margins in patients. Though our tumors expressed EGFR abundantly on the cell surfaces, as did those in other publications, expression is variable and could lead to false negatives. Longitudinal clinical studies are needed to assess margin status and recurrence of ameloblastoma in future human trials. We believe the concepts described in this manuscript, once translated into the clinical setting as in other human tumors, will allow surgeons to more confidently excise ameloblastomas by accurately assessing tumor margins and preserving normal tissue, thereby improving long-term local tumor control and, ultimately, patient outcomes.

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Figure 1.

(A) EGFR expression in ameloblastoma (AB) and squamous cell carcinoma cells. Relative gene expression of EGFR was determined by qRT-PCR. The housekeeping gene GAPDH was used as normalizing reference. All the experiments were performed in duplicate and repeated two times (error bars denote SD). (B) Cetuximab-IRDye800 (Cetux) binds to ameloblastoma cells, while control IgG-IRDye800 (IgG) did not. Cells are plated on transwell glass slides, labeled with antibodies overnight, and imaged on the Odyssey system. (C) EGFR protein expression in cases of ameloblastomas. Protein expression was determined by immunohistochemistry using an EGFR specific antibody (10X).

Figure 2.

In vivo labeling of ameloblastoma (AB-20) tumor tissue. TBR was significantly higher in the cetuximab-IRDye800-treated animals compared to the IgG-IRDye800-treated animals (*p < 0.05, ** p < 0.01). Error bars are standard error. Representative images obtained using the Pearl imaging system.

Figure 3.

In vivo labeling of ameloblastoma tumor tissues pre-resection. The skin flap overlying the implanted tumors was removed and animal were imaged. Representative images obtained using the Pearl imaging system. The TBR were significantly higher in the cetuximab-IRDye800-treated animals compared to the IgG-IRDye800-treated animals (* $p < 0.05$, ** $p < 0.01$). Error bars are standard error.

Figure 4.
Small amounts of tumor tissue are detectable when labeled with Cetuximab-IRDye800.
Post-resection tumor tissue is weighed and placed into the wound bed for imaging.

Figure 5.

Ameloblastoma tumor tissue is confirmed in implants post-resection. Surgical tissue is implanted subcutaneously in mice for six –eight weeks. Upon resection, tumor tissue is processed and paraffin embedded, stained for hematoxylin and eosin (H&E), and used for pathologic assessment.

Table 1.

Current uses of fluorescent antibody-based optical imaging for surgical navigation

Current use of Antibody-based Fluorescent Surgical Navigation			
Tumor Site/Type	Antibody	Receptor Target	Reference
Breast	Panitumumab	EGFR	18
	Tocilizumab	IL-10	18
	Bevacizumab	VEGF-A	19
Colorectal	Panitumumab	EGFR	20
	Bevacizumab	VEGF-A	21
	Labetuzumab	CEA	22
	SGM-101	CEA	23
	MN-14	CEA	24
	ATN-658	uPAR	25
Cutaneous SCC	Panitumumab	EGFR	26
	Bevacizumab	VEGF-A	26
Glioma	Cetuximab	EGFR	27
	Cetuximab	EGFR	28
	Panitumumab	EGFR	29
	MMP-750	MMPs	30
HNSCC	Cetuximab	EGFR	31–37
	Panitumumab	EGFR	38
	BIWA	CD44v6	39
	ATN-658	uPAR	40
Hepatocellular	YY146	CD146	41
Melanoma	Panitumumab	EGFR	42, 43
	mAb62	Kv10.1	44
	Toclizumab	IL-10	42
Ovarian	IntegriSense 680	$\alpha v\beta 3$ -integrin	45
	Bevacizumab	VEGF-A	46
	COC183B2	COC183B2	47
Pancreatic	Cetuximab	EGFR	48
	SGM-101	CEA	49
	hM5A	CEA	50
Prostate	mAb 7.4	EpCAM	51
	A11 Mb	PSCA	52
Renal	Girentuximab	CAIX	53, 54
Sarcoma	Cetuximab	EGFR	55

Abbreviations: CAIX, carbonic anhydrase IX; CEA, carcinoembryonic antigen; EGFR, epidermal growth factor receptor; EpCAM, epithelial cell adhesion molecule; HNSCC, head and neck squamous cell carcinoma; IL-10, interleukin 10; Kv10.1, ether-à-go-go voltage-gated potassium channel; MMP, matrix metalloproteinase; SCC, squamous cell carcinoma; PSCA, prostate stem cell antigen; uPAR, urokinase-receptor; VEGF-A, vascular endothelial growth factor A