Published in final edited form as:

Proc SPIE Int Soc Opt Eng. 2019 February ; 10853: . doi:10.1117/12.2510897.

Paired-agent imaging for detection of head and neck cancers

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Abstract

Head and neck cancers overwhelmingly overexpress epidermal growth factor receptor (EGFR). This overexpression has been utilized for head and neck cancers using molecular targeted agents for therapy and cancer cell detection. Significant progress has been made in using EGFR-targeted fluorescent antibody and Affibody molecule agents for fluorescent guided surgery in head and neck cancers. Although success in achieving tumor-to-background ratio of 3–5 have been achieved, the field is limited by the non-specific fluorescence in normal tissues as well as EGFR specific fluorescence in the oral cavity. We propose that paired-agent imaging (PAI) could improve the contrast between tumor and normal tissue by removing the fluorescent signal arising from non-specific binding. Here, ABY-029 – an anti-EGFR Affibody molecule labeled with IRDye 800CW – and IRDye 680RD conjugated to Affibody Control Imaging Agent molecule (IR680-Aff_{ctrl}) are used as targeted and untargeted control agents, respectively, in a panel of head and neck squamous cell carcinomas (HNSCC) to test the ability of PAI to increase tumor detection. Initial results demonstrate that binding potential, a value proportional to receptor concentration, correlates well to EGFR expression but experimental limitations prevented pixel-by-pixel analysis that was desired. Although promising, a more rigorous and well-defined experimental protocol is required to align *ex vivo* EGFR immunohistochemistry with *in vivo* binding potential and fluorescence intensity. Additionally, a new set of paired-agents, ABY-029 and IRDye 700DX, are successfully tested in naïve mice and will be carried forward for clinical translation.

Keywords

head and neck cancer; paired-agent imaging; epidermal growth factor receptor; Affibody molecule; IRDye 800CW; ABY-029; IRDye 680RD; IRDye 700DX; Affibody Control Imaging Agent; fluorescence guided surgery

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1. INTRODUCTION

Despite advancements in detection and treatment, the survival rate for patients with HNSCC (oral cavity, nasopharynx, other pharynx, larynx) has remained poor with recurrence rates as high as 50% [1]. HNSCC is considered a locoregional disease; however, the inability to control primary tumor spread is the leading cause of death for these patients [2]. Adjuvant chemotherapies and radiotherapies to treatment protocols have not significantly increased the patient's long-term survival rate and obtaining clear margins during surgery remains critical for positive patient outcomes [3]. A retrospective study by Binahmed *et al.* (2007) [4] demonstrated that patients with involved margins had a 38% 5-year survival rate, compared to 58% of patients with tumor cells within 2 mm of the margin and 69% with clear surgical margins. Overall, detection of tumor at or close to the surgical margins increased the risk of death at 5-years by 90%.

Complete resection of HNSCC tumors using wide surgical margins has a large positive impact on patient survival. However, those patients with long survival times often suffer from significant and debilitating morbidity [5]. This is especially so in oral HNSCCs due to the inherent complexity of physiologic functions (respiration, olfaction, taste, speech, etc.). Patients undergoing oral tumor resections run the risk of experiencing many functional and cosmetic impairments including: dysphagia, lip contracture, impaired dental closure, vocal cord immobility, swallowing function deficiency, tracheal stenosis, swelling and lymphedema, reduced ability to swallow, and respiratory insufficiency [6]. In addition, 70% of patients that undergo surgical resection for HNSCC report neck and shoulder pain, neuropathic pain of the neck, myofascial pain, joint pain and lack of sensation in the neck [7]. These sometimes debilitating impairments can last a lifetime, reduce quality of life and inhibit everyday functions.

Although promising, single fluorescence agents targeting epidermal growth factor receptor (EGFR) are qualitative at best, have moderate tumor-to-normal tissue contrast, and have not yet demonstrated long-term improved patient outcomes. Our extensively developed *in vivo* paired-agent imaging method (Figure 1) allows quantification of extracellular receptor expression (and identification of cancerous tissue) by utilizing simultaneously delivered targeted and non-targeted (*i.e.*, perfusion) agents to reference the plasma delivery and leakage [8–26]. The ability to eliminate the non-binding fluorescence contribution in the tumor and surrounding normal tissues increases the observed tumor-to-normal tissue contrast and improves our ability to observe microscopic tissue burden. We have validated PAI using two different paired-agent sets: **1)** IRDye 800CW-epidermal growth factor and IRDye 700DX [27]; and **2)** ABY-029 (anti-EGFR Affibody molecule labeled with IRDye 800CW, Food and Drug Administration (FDA) exploratory Investigational New Drug (eIND; 122681) and Affibody Control Imaging Agent molecule labeled with IRDye 680RD (IR680-Aff_{ctrl}) [28]. Additionally, we have demonstrated that the PAI signal is equivalent to *ex vivo* immunostaining techniques [28], and can detect fewer than 200 tumor cells non-invasively in breast cancer draining lymph nodes [29]. Cellular resolution could potentially be increased to even fewer cells when imaging at, or near, the tissue surface within a tumor bed as is proposed here. These promising results suggest that PAI is capable for distinguishing HNSCC from normal tissues during surgery.

2. MATERIALS AND METHODS

2.1 Cell culture

Head and neck squamous cell carcinomas SSC-25, SCC-15, and SCC-9 were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured according to their culture methods. Briefly, a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium was used. Additionally, the medium contained 1.2 g/L sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES and 0.5 mM sodium pyruvate. The medium was supplemented with 400 ng/mL hydrocortisone and 10% fetal bovine serum (FBS).

2.2 Quantitative flow cytometry

The number of epidermal growth factor receptors (EGFR) available for binding per cell was determined using quantitative flow cytometry, as described previously [27, 28]. Briefly, when the cells reached 80% confluence they were incubated in 4 mg/mL EGF Biotin (Thermo Fisher Scientific, Inc.) for 30 minutes, washed with phosphate buffered saline (PBS) and then secondarily labeled with 1:25 dilution of Cy5-streptavidin (Thermo Fisher Scientific, Inc.). Control cells were stained only with the secondary Cy-5 streptavidin to account for autofluorescence and nonspecific staining. Quantification was performed by standardization with Quantum Cy5 MESF beads, used as described by the manufacturer (Bangs Laboratory). Flow cytometric data were acquired with an 8-color MACSQuant-10 (Miltenyi Biotec) and analyzed using FlowJo (FlowJo, LLC). The fluorescence geometric mean of the control cells was subtracted from the geometric mean of all the EGF-stained cells. The number of receptors per cell was calculated assuming one molecule of biotin per EGF and three Cy5 fluorophores per streptavidin molecule, as specified by Invitrogen.

2.3 Tumor implantation

All animals were used in accordance to the approved protocols from the Institutional Animal Care and Use Committee (IACUC) at Dartmouth College. Cells were prepared for implantation in 1×10^6 cells per 25 μ L in whole culture medium and then mixed with an equal volume of low growth factor Matrigel® for a total implantation volume of 50 μ L containing 1×10^6 cells. Mice were anesthetized (1 L/min O₂, 1–2.5% vaporized isoflurane) and tumors implanted onto the right flank by tenting the skin with forceps and injecting 50 μ L into the subcutaneous space. Tumors were grown for 2–4 weeks until they reached an approximate volume of 250 mm³, measured with calipers and volume calculated using the equation for the volume of an ovoid.

2.4 Paired-agent imaging

The day of imaging, the mice were anesthetized to a surgical plane using ketamine:xylazine (100:10 mg/kg). The skin over the tumor and surrounding normal tissues was surgically removed to expose the tumor and muscle. The mice were placed on a slide with piece of black fabric with a 1 $\times$ 2 cm rectangle cut into it, such that only the tumor, leg muscle and skin were visible through the rectangle. The mouse was secured to the glass slide using surgical tape and placed in an Odyssey CLx fluorescence scanner. The 700 and 800 channel

lasers were used in Manual mode at a setting of 0.5, 1-mm offset, medium scan quality, and 84 μm resolution. The total scan time for both the 700 and 800 channel was under 1 minute. An autofluorescence image was collected prior to injection. Each mouse was simultaneously administered a 1:1 molar ratio (0.1 nmole each) of ABY-029, an anti-EGFR Affibody molecule labeled with IRDye 800CW, and IRDye 680RD conjugated to Affibody Control Imaging Agent (IRDye 680-Aff_{ctrl}) in 200 μL of phosphate buffered saline (PBS). Imaging was immediately started, and an image was collected every minute for 1-hour. After imaging each mouse was sacrificed using cervical dislocation and placed in formalin to preserve tissue location. Naïve mice were used to image ABY-029 and IRDye 700DX carboxylate as an alternative paired-agent set.

2.5 Pathology

After 24-hours in formalin, the glass slide was removed, and pins dipped in surgical ink were pushed into the tissue around the opening in the black fabric to create markers to be used for tissue alignment. The black fabric was then removed, and the tissue surrounding the imaged area was removed, placed in a histology cassette, and placed back in formalin for pathology. The tissues were sectioned at 4 μm and consecutive sections were stained for H&E and EGFR immunohistochemistry (IHC). H&E and EGFR IHC slides were digitized using a Vectra 3 scanning microscope (Perkin Elmer).

2.6 Image analysis

Binding potential (BP) maps of EGFR concentration were created using Matlab code created based on Tichauer et al (2012) [27] and Samkoe et al (2014) [28], which required deconvolution of the non-targeted control agent. The targeted fluorescence (ABY-029), untargeted fluorescence (IRDye 680-Aff_{ctrl}), and BP maps are naturally aligned. The fluorescent and BP were aligned to the EGFR IHC images using Matlab. Both rigid and non-rigid transformations were performed by selecting corresponding control points on the BP map and EGFR IHC images. Region-of-interest (ROI) masks were created over tumor, muscle, and skin and both the average signal and individual pixels in each region was compared between fluorescence signal, BP, and EGFR stained IHC. Images were blurred using *impyramid* function and pixel values were compared for each condition over 5 levels of blurring.

2.7 Statistical analysis

Pearson linear correlation was performed between fluorescence intensity or BP map and EGFR IHC stain intensity. Pearson correlation value, r , was considered a strong positive if $1 > r > 0.5$, moderate positive if $0.5 > r > 0.3$, weak positive if $0.3 > r > 0.1$, no correlation if $0.1 > r > -0.1$, weak negative if $-0.1 > r > -0.3$, moderate negative if $-0.3 > r > -0.5$, or strong negative if $-0.5 > r > -1$.

3. RESULTS AND DISCUSSION

The goal of tumor-free margin assessment during surgical resection of oral head and neck squamous cell carcinomas (HNSCC) is to increase patient survival and decrease morbidity. However, clear margins are difficult to achieve due to the inherent complexity of

physiological structures, which cause disfiguring and debilitating morbidity. Human trials have been performed using a single fluorescent targeted antibody for resection using fluorescent guided surgery (FGS) of HNSCC [30]. Although promising, single antibody imaging suffers from long injection-to-resection time, and modest contrast observed using tumor-to-normal tissue background ratio ($TBR = 4.3$) due to a complex combination of receptor binding, vascular density and perfusion, and inhibited lymphatic clearance [30]. A ‘cold dose’ (sub-therapeutic dose of naive antibody) has been administered prior to the imaging antibody to deplete endogenous receptor ‘sinks’ and increase contrast [31]. This has been met with limited further enhancement ($TBR = 5.5$) [31]. Currently, three major obstacles limit wide-spread adaptation of FGS in HNSCC: 1) high normal tissue signal; 2) long administration-to-surgery times for antibody agents; and 3) demonstration of improved patient outcomes over traditional resection.

Previously, we have demonstrated the feasibility of PAI using *in vitro* assays, and in *in vivo* subcutaneous murine models. These PAI experiments have demonstrated: **1)** binding potential scales linearly with EGFR expression both *in vivo* and *in vitro* [27], **2)** there is a high correlation measured with Pearson’s coefficient between binding potential and *ex vivo* EGFR immunohistochemistry [28], and **3)** that fewer than 200 tumor cells can be detected non-invasively in breast cancer draining lymph nodes [29]. Cellular resolution could potentially be increased to even fewer cells when imaging at, or near, the tissue surface within a tumor bed as is proposed here. We have validated PAI using two different paired-agent sets: **1)** IRDye 800CW-epidermal growth factor and IRDye 700DX [27]; and **2)** ABY-029 (anti-EGFR Affibody molecule labeled with IRDye 800CW, Food and Drug Administration (FDA) exploratory Investigational New Drug (eIND; 122681) and Affibody Control Imaging Agent molecule labeled with IRDye 680RD (IR680-Aff_{ctrl}) [28].

Here, we used the same Affibody molecule combination as previously published in Samkoe, et al (2014) [28] – ABY-029 and IR680-Aff_{ctrl} as shown in Figure 2. Binding potential (BP) maps were created using the kinetic PAI model (Figure 1), as previously described [28]. The digitized EGFR immunohistochemistry section was color deconvolved to retain only the brown staining and then is compared to the fluorescent images (ABY-029 and IR680-Aff_{ctrl}) and the CP map. The example provided in Figure 2 is of an SCC-15 tumor, in which successful alignment could be achieved. Spatial alignment between the fluorescent images or BP map with immunohistochemistry stained sections was difficult due to the topography of the sample and several distinct tissue types in the image.

As with previous studies, we found that in the three HNSCC tumor lines tested that *in vitro* EGFR expression was well matched to *in vivo* EGFR concentration determined by binding potential (Figure 3). However, this was based on a bulk average of the tumor and as can be noted in Figure 3, the expression of EGFR was not homogeneous throughout the tumors. Therefore, we attempted to align the *in vivo* BP and fluorescent images with the *ex vivo* EGFR immunohistochemistry slides (Figure 2). For several samples in which satisfactory alignment could be achieved (Figures 1 and 4) we found that there was a strong positive correlation between the EGFR IHC and BP (Figure 4). In addition, at 1-hour post-administration of the paired-agents there was a moderate and strong, negative correlation between BP and non-targeted and targeted fluorescence, respectively (Figure 4). However, in

the vast majority of samples satisfactory tissue alignment was not possible due to several factors involving the subcutaneous model. Multiple tissue types existed in the region-of-interest (tumor, muscle, and skin) but they did not all exist in the same 3-dimensional plane. As such, there were large gaps between tissue types, tearing or tissue rotation relative to the tumor, and in some cases, skin was not visible at all. This hindered the alignment process, as the ink spots that were imprinted in the fixed tissue did not readily appear in the tissue sections, and when they did they were often grossly misaligned. However, the samples that did align well, including the SCC-15 tumor shown in Figure 2 and analyzed in Figure 4 give good promise to PAI for pixel-to-pixel determination of EGFR concentration *in vivo*.

Moving forward, the subcutaneous model will be abandoned and the HNSCC tumor cell lines will be implanted in the tongue of mice. The tongue is relevant for HNSCC and may be an orthotopic model in some cases. In addition, the tongue provides a rigid tissue structure to aid in alignment and ease the transfer of tissue from *in vivo* to *ex vivo* pathology.

The key factors when choosing paired-agents to ensure that the path to clinical translation is as simple as possible, while having the largest impact on all HNSCC patients include: **1)** The paired-agents should be available in FDA-approved form or in “good manufacturing practice” (GMP) form; **2)** Paired-agents should be well matched in respect to plasma curves (**Figure 2A**) and pharmacokinetics within normal tissue devoid of the receptor of interest (i.e., muscle, **Figure 2C**); **3)** The paired-agent receptor binding kinetics are similar *in vitro* and *in vivo*, such that the number of receptors reported is accurate; and **4)** The absolute concentration and spatial distribution of the target receptor in normal and tumor tissues is accurately described by PAIRS, such that clear delineations can be made between tumor vs. normal cells. We have demonstrated that these factors are indeed addressable within the time frame of this grant.

We have chosen ABY-029 and IRDye 700DX are good candidates for BP relevant to head and neck cancers. ABY-029 is a synthetic anti-EGFR Affibody molecule that is covalently bound to IRDye 800CW, is an FDA-approved exploratory Investigational New Drug (eIND, 122681), has been GMP produced for a Phase 0 clinical trial ([ClinicalTrials.gov](https://clinicaltrials.gov) Identifier) currently in progress, and has been rigorously tested in our laboratory for toxicity [32] and as a targeted PAI agent [28]. A cetuximab and IRDye 700DX conjugate has been developed extensively as a photoimmunotherapy agent [33–36] and is currently in clinical trial for head and neck cancers ([ClinicalTrials.gov](https://clinicaltrials.gov) Identifier). As part of this head and neck clinical trial, the unconjugated IRDye 700DX will be administered and monitored for up to 14-days in a subset of patients to determine various pharmacokinetic parameters. Preliminary data was collected using naïve mice and a 1:1 mixture of ABY-029:IRDye 700DX (**Figure 2C & D**) and demonstrates that: 1) the *in vivo* tissue uptake and distribution curves closely match, 2) binding potential is close to zero in muscle, which is devoid of EGFR, and low in other normal tissues, and 3) the BP studies utilizing IRDye 800CWEGF and IRDye 700DX, and ABY-029 and IR680-Aff_{ctrl} are highly correlated [28], with a slope of nearly one, suggesting the paired-agents are interchangeable. The preliminary data and the clinical applicability of both ABY-029 and IRDye 700DX suggests these are strong candidate paired-agents for human PAIRS.

4. CONCLUSIONS

Paired-agent imaging is a promising technique to reduce the contribution of non-specific fluorescent signal arising from normal surrounding tumors and non-specific uptake in tumors due to the enhanced permeability and retention effect. We demonstrated that EGFR expression measured *in vitro* using quantitative flow cytometry, *in vivo* using PAI binding potential, and *ex vivo* EGFR immunohistochemistry and are well correlated in three HNSCC cell lines with varying levels of EGFR expression. However, failed experimental protocols for aligning subcutaneous tissues with *ex vivo* immunohistochemistry staining prevented meaningful analysis of the data on a microscopic scale, as desired. As such, a new model for HNSCC tumors implanted in the tongue will be investigated to aid in image alignment. The paired agents of ABY-029 and IR680-Aff_{ctrl} worked well for determining EGFR concentration *in vivo* but are not realistic pair of agents for clinical translation. Therefore, moving forward ABY-029 and IRDye 700DX carboxylate will be investigated for more relevant clinical translation.

ACKNOWLEDGEMENTS

The authors of this work would like to acknowledge the following funding sources: National Cancer Institute Early Stage MERIT Award R37 CA212187, Dartmouth College's SYNERGY Translational Pilot Project Award, and the Department of Surgery at Geisel School of Medicine's Harmes Scholar Award.

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Figure 1.

The paired-agent imaging (PAI) approach uses tissue transport of two fluorescent agents to quantify EGFR expression: 1) an untargeted Control Agent (ROI_C); and 2) an extracellular receptor Targeted Agent (ROI_T). K_1 and k_2 are the rates of extravascular leakage and tissue efflux, respectively; & k_3 and k_4 , “on” and “off” rates of targeted tracer binding. Kinetic PAI: The Lammertsma equation where R_1 is equation to K_1/K_1' . Single Time Point PAI: describes the calculation of BP with single time point images at times >30 min post-injection. Binding Potential (BP): The key parameter, k_3/k_4 , is proportional to B_{avail} , the concentration of available binding sites, and k_A , the receptor-target binding affinity (inversely proportional to k_D , the receptor-target dissociation constant).

Figure 2.

Experimental image collection for SCC-15 tumor demonstrating the experimental flow.

Mice with subcutaneous SCC-15 tumors were injected with ABY-029 and IRDye 680RD-Affibody Control Imaging Agent. Fluorescent images were collected on the Odyssey CLx over an hour. The final images collected at 60 minutes are shown here for both ABY-029 and IRDye 680RD-Aff_{ctrl}. The corresponding and inherently aligned binding potential (BP) map is shown next to the aligned EGFR IHC performed in Matlab. The EGFR IHC image was color separated to isolate the brown stain only, and this brown stain was compared to the BP map, ABY-029 fluorescence and IRDye 680RD-Aff_{ctrl} fluorescence.

Figure 3.

EGFR expression measured *in vitro* correlates with *in vivo* BP measures. **A)** EGFR molecules per cell were quantified *in vitro* in five HNSCC cell lines using quantitative flow cytometry and are compared to the ‘gold standard’ of EGFR producing cell line, A431. **B)** High (SCC-15), moderate (SCC-25) and low (SCC-9) EGFR expressing cell lines were measured *in vivo* using PAI to determine the BP, which is proportional to EGFR concentration. **C)** The *in vitro* EGFR receptors per cell are plotted against the *in vivo* BP and a linear regression demonstrates that the two measures of EGFR correlate well.

Figure 4.

Correlation of EGFR stain intensity from immunohistochemistry with binding potential (*left*) and targeted ABY-029 (green, *right*) and non-targeted IR680-Aff_{ctrl} (red, *right*). BP displays a strong positive linear correlation with EGFR expression, while ABY-029 and IR680-Aff_{ctrl} display moderate and weak, negative linear correlations, respectively, with EGFR.

Figure 5.

Selection of clinically relevant agents for PAI. **A)** ABY-029 and IRDye 700DX are both in clinical trials and display very similar plasma excretion curves. **B)** Previous studies [27, 28] utilized EGF-IRDye 800CW and IRDye 700DX [27], and anti-EGFR Affibody molecule labeled with IRDye 800CW and IR680-Aff_{ctrl} [28]. The binding potential calculated in the same 4 tumor lines are linearly correlated with a strong, positive Pearson's correlation ($r = 0.98$). **C)** The normalized fluorescence uptake in normal tissues (muscle, skin, fat) are similar. **D)** The binding potential map of normal tissue in a naïve mouse indicates that ABY-029 and IRDye 700DX be success paired-agents.