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MRI-coupled fluorescence tomography quantifies EGFR activity in brain tumors

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Abstract

Rationale and Objectives—This report demonstrates the diagnostic potential of MRI-coupled fluorescence molecular tomography (FMT) to determine epidermal growth factor receptor (EGFR) status in brain cancer.

Materials and Methods—Two orthotopic glioma xenograft models were used in this study, one representing high EGFR expression and the other low expression. Nude mice were inoculated with cells from either one of the tumor lines or were used in sham surgery control group. Animals were imaged using a unique MRI-FMT scanner 48 hours after intravenous injection of a near-infrared fluorophore bound to epidermal growth factor (EGF) ligand. Coronal images of fluorescence activity of the injected dye in the mouse brain were recovered using the MRI images as anatomical templates.

Results—*In vivo* images of fluorescence activity showed significant differences between animal populations, an observation confirmed by ROC analysis which revealed 100% sensitivity and specificity between animal groups implanted with EGFR⁽⁺⁾ and EGFR⁽⁻⁾ tumor lines. Similar performance was observed between EGFR⁽⁺⁾ and sham surgery control animals.

Conclusion—This pre-clinical study suggests that MRI-FMT with fluorescent EGF provides excellent discrimination between tumors based on EGFR status. Reliable quantification of receptor status using minimally-invasive techniques would be an important innovation for investigating new and existing cancer treatments that target these cellular mechanisms in research animals and may be applied to identify receptor amplification in human brain cancer patients. This study represents the first systematic multi-animal validation of receptor specific imaging using MRI-guided fluorescence tomography.

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Epidermal growth factor receptor (EGFR) is a cell receptor known to be amplified in a large percentage of cancers, 55% by some estimates, and contributes to malignant cell proliferation when activated via binding by an associated ligand, such as epidermal growth factor (EGF) (1-4). Shutting down this pathway to halt malignant proliferation has been the subject of many research and drug development efforts in recent years and several EGFR-targeted therapies are now commonly used against some forms of cancer(3); however, the ability to image EGFR activity has not been widely examined in a way which would translate to human use. The pre-clinical study reported here examines the diagnostic potential of MRI-coupled fluorescence molecular tomography (FMT) to determine EGFR status between two different brain tumor cell lines; U251 (human glioma) and 9L-GFP (rat gliosarcoma transfected with green fluorescent protein). *In vitro* studies using flow cytometry have previously demonstrated that the U251 tumors have a 20-fold higher uptake of EGF as compared to the 9L-GFP line, suggesting that EGFR expression is greatly amplified in this tumor cell line(5). Thus, these two cell lines were classified as either EGFR⁽⁺⁾ or EGFR⁽⁻⁾ for the purpose of this study.

FMT is a minimally invasive imaging modality for studying the underlying biology of disease in living tissue. Pre-clinical studies in murine models make up the bulk of the *in vivo* FMT studies reported in the literature, which includes efforts to quantify enzyme activity in atherosclerotic inflammation(6) and gliomas(7), myocardial macrophage infiltration(8), bone regeneration(9), drug sensitivity in lung carcinoma(10), and other tumor-specific mechanisms in lung(11), breast (12-13) and brain tumors(11-14-15). This modality is well suited for investigating processes associated with transmembrane protein receptors that are frequently amplified in tumor cells. The higher receptor density on malignant cells provides a natural contrast mechanism for molecular probes with high affinity for the protein binding sites. Exploiting this abnormal biology with FMT could help identify tumors with amplified receptor status, monitor the binding capacity of therapeutic drugs, and track overall tumor burden. This is especially effective when the demonstrated benefits of FMT are combined with structural-based information provided by conventional imaging tools, as is done herein.

Materials and Methods

Fifteen animals were included in this MRI-FMT study, six with U251 tumors, five with 9L-GFP tumors, and four tumor-free controls. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC). Prior to imaging, each nude mouse underwent intracranial surgery during which tumor cells were implanted in the animal's brain. Tumors were allowed to grow for between 14 and 23 days. Control animals underwent the same surgical procedure but received PBS injections without tumor cells. Every animal received an injection containing 1 nmole of Licor IRDye-800CW EGF (LI-COR Biosciences, Lincoln, NE) in the tail vein 48 hrs prior to the scheduled imaging time. This imaging agent is composed of an NIR fluorescent dye conjugated to the epidermal growth factor ligand (EGF).

The MRI-FMT imaging system has been described in a previous publication(14) and primarily consists of a Philips 3T clinical MRI scanner, a specialized rodent MRI radio frequency (RF) coil designed to accommodate eight optical fibers in a circular array, and a parallel detection spectrometer-based fluorescence tomography imaging system. During the scan, the optical instruments reside outside of the magnet room with long optical fibers coupling light between the source and detectors, and animal tissue. This unique arrangement allows for the simultaneous acquisition of optical and MRI data, facilitating straightforward image co-registration. The animal interface has been modified since details of the instrument were originally published(14) by replacing the mouse head holder with a hollow cylinder made of black acetyl, shown circumscribing the animal's head in Fig. 1(a). This prevents remitted light exiting the tissue from reflecting back into the imaging volume and detection fibers. The animal interface was also incorporated with 11 MRI sensitive fiducials which help locate the otherwise

MR-invisible fibers in the images. In prior work we demonstrated the system's imaging capabilities using tissue-simulating phantoms and a single animal test case. Herein, the diagnostic capabilities of the system are challenged in a systematic multi-subject animal study which takes into account the significant biological variability associated with live tissue imaging.

For *in vivo* imaging, anesthetized (1.5% isoflurane, 1 L/min oxygen) mice were positioned in the coil such that the coronal plane of the optical fibers aligned with the mid-point between the eyes and the ears, as depicted with a rendered surface in Figure 1(b). Before optical measurements were made, preliminary MRI scans were acquired to ensure that the optical fiber plane intersected the bulk of the tumor. In some cases, animals were repositioned and the procedure repeated until the alignment was satisfactory, at which time the full MRI-FMT scan was initiated. The MRI image series' acquired for each animal included T2 weighted images and T1 weighted images before and after the administration of gadolinium contrast, and were completed in about 23 minutes. This provided ample time for the optical system to acquire the full complement of fluorescence and excitation measurements for all 56 source-detector pairs. The entire imaging process including time for anesthetic induction could be completed in 30 minutes for one animal.

Within an hour after the scan, animals were sacrificed and processed for *ex vivo* analysis. In most cases, the brains were surgically extracted and sliced into sections for *ex vivo* fluorescence scanning on a Licor Odyssey scanner. Some animals were frozen to -80° C and the head sectioned into approximately 4mm thick slices before being scanned on the Odyssey system. In either case, the *ex vivo* imaging was completed within 2 hours of the *in vivo* scans and prior to fixation in formalin. After *ex vivo* fluorescence scanning, tissues were fixed and stained for pathological analysis.

Panels (c) through (h) in Fig. 1 illustrate the procedure used to recover fluorescence tomography images from the acquired data. First, the contrast-enhanced MRI image which coincided with the plane of the optical fibers was identified. In the example in Fig. 1, this corresponds to the image in panel (e). With the exception of control mice, animals that either did not show any Gd contrast-enhancement or showed enhancement outside of this plane were excluded from the analysis. Next, the fiber positions were located in the image with reference to the MR-sensitive fiducials. The selected MR image was then segmented into several regions including the brain, the Gd-enhanced regions in the brain, any other abnormal-looking features in the brain, and the rest of the tissue outside of the brain, as shown in Fig. 1(g). MR images of the control mice which showed no abnormal features in the brain were segmented into two regions consisting of the brain and the surrounding tissue. The segmented mask was then used to generate a Finite Element Method (FEM) mesh for reconstructing images of fluorescence yield in the tissue based on the diffusion approximation(16-17). Fluorescence yield is defined as the product between the fluorescence quantum yield and the absorption coefficient of the imaging agent at its excitation wavelength and thus is related to the concentration of the drug. Optical properties from the literature(18) were assigned to the brain and the rest of the tissue in the mesh as described in Ref (14). The previously described data calibration procedure made use of a spectral unmixing algorithm to decouple the dye signal from the contaminating tissue autofluorescence signal. This technique fits pre-recorded spectra of the dye and tissue autofluorescence to determine the intensity of each signal in a given measurement, a process unique to spectrally-resolved FMT scanners. Images of fluorescence yield were then recovered using the laplacian-type regularization implementation(17) and overlaid on the simultaneously acquired MRI image, as demonstrated in Fig. 1 (h).

Results

MRI-FMT images revealed that elevated levels of fluorescence activity in the U251 population corresponded to Gd-enhanced tissue regions in the MRI images. Figure 2 (a) and (b) show examples of FMT images overlaid on their corresponding T1-weight MRI images for the U251 and 9L-GFP tumor lines, respectively. These images are plotted on the same color scale and show highly elevated levels of fluorescence activity in the U251 tumor and little to no activity in the 9L-GFP tumor. While the *ex vivo* fluorescence images of the same animals shown in (c) and (d) confirm the presence of elevated fluorescence activity in the brain, they were neither rigorously registered with the *in vivo* images nor calibrated against a fluorescent standard and therefore cannot directly validate the FMT results. Similarly, histopathology slides like those shown in (e) and (f) were used to confirm only the general location and morphology of the malignant growth in the brains. Based on these criteria, the two image series' in Fig. 2 demonstrate acceptable agreement between the three modalities.

The images in Fig. 2 (a) and (b) are representative of the entire image set. Qualitative examination of the images definitively shows significantly higher levels of fluorescence yield in Gd-enhanced regions of the brain in the U251 populations than in either the enhanced regions of the 9L-GFP population or in the brains of control mice. Plotted on a consistent color scale bound by the minimum and maximum values amongst the entire image set (not shown), all U251 tumor regions are easily discerned while the 9L-GFP and control group images appear almost completely dark with the exception of one animal in the 9L-GFP group which shows discernable contrast in the tumor region. These observations were confirmed by quantitative analysis of the images using the mean values of fluorescence yield in the tissue regions enhanced by gadolinium contrast. The mean value of the entire brain region was used for control animals since there were no discernable abnormalities in the brain. The results are plotted in Fig. 3 (a) and confirm the qualitative observations. The fluorescence activity in U251 tumors is shown to be much higher than in 9L-GFP tumors and higher than the brains of control mice. Statistical significance was found between the U251 and control groups as well as the U251 and 9L-GFP groups based on a one-tailed t-test (p-values for both < 0.012).

The diagnostic performance of the system was determined using Receiver Operator Characteristic (ROC) curves between the different populations. These curves were calculated using the mean value of fluorescence yield in tissue regions enhanced by gadolinium contrast for the tumor-bearing animals, and in the entire brain region for the control mice. The area-under-the-curve (AUC) values are tabulated in Fig. 3 (b) and show perfect sensitivity and specificity values when comparing the U251 and 9L-GFP groups, and the U251 and control groups, while the system provides virtually no diagnostic capacity between 9L-GFP and control groups.

Discussion

MRI-guided fluorescence tomography demonstrated excellent diagnostic capability between EGFR⁽⁺⁾ and EGFR⁽⁻⁾ tumor lines and between EGFR⁽⁺⁾ and control animals *in vivo*. Differences in fluorescence yield corresponding to Gd-enhanced regions in the brain were statistically significant between EGFR⁽⁺⁾ and EGFR⁽⁻⁾ tumor lines and between EGFR⁽⁺⁾ and control animals, even though the sample sizes in each group were relatively small. This translated to perfect ROC performance. These results are encouraging and show great potential for fluorescence imaging of receptor status *in vivo*.

Maintaining the diagnostic performance between tissues with very small differences in receptor activity will be more challenging. This is evidenced by examining the performance between the EGFR⁽⁻⁾ and control animals. Differences between EGFR⁽⁻⁾ and control animals were not

statistically significant and produced failed ROC performance tests, with an AUC = 0.55. While this is not unexpected given the very low receptor status of 9L-GFP tumor cells measured *in vitro*(5), the cell line does express EGFR to some extent and *ex-vivo* images of the 9L-GFP line showed a modest amount of fluorescence in the tumor region. The results indicate that these small changes in fluorescence activity are below the diagnostic limits of the imaging system. Ongoing studies similar to this one will help establish these limits in the context of potential bias from the imaging technique itself and biological variability.

Synthesizing data between MRI and optical modalities in the manner described in this study raises legitimate concerns that the MRI data over-constrains the optical image recovery process. This has been addressed in previous work(14- 17) using simulated and phantom data, and while it was not investigated directly in this study, including both EGFR positive and negative tumor lines verifies that the structural information from the MRI used in the optical image recovery process does not inappropriately bias the diagnostic information provided by the *in vivo* fluorescence images. In the imaging paradigm deployed here, any Gd-enhanced regions in the MRI images were segmented into suspicious regions and incorporated in the optical imaging algorithms. Including a group of animals inoculated with the EGFR⁽⁻⁾ tumor cell line was important to demonstrate diagnostic specificity in animals with compromised blood-brain barriers, a condition not found in control animals. Gadolinium-enhanced regions that identify EGFR⁽⁻⁾ tumors can be thought of as false positive readings which the FMT data correctly characterized as having little EGFR activity. In a more broad application, this added specificity can be extrapolated to distinguishing between malignant and benign lesions. The data presented here strongly suggest that this is the case, though biological factors which may have had an overwhelming influence on the results must be considered.

While the data are generally compelling, the influence of drug pharmacokinetics on the tumor-to-normal tissue contrasts *in vivo* is a potential confounding factor that may have had a substantial impact on the results. In most tumor masses, the different vascular structures found between malignant tumors and normal tissue provides inherent contrast even when non-targeted contrast agents are injected and imaged over time. This phenomenon, known as the EPR effect (enhanced permeability and retention), dominates the tissue contrast shortly after administration, even when highly specific imaging molecular probes are used. The Gd-MRI images in this study revealed noticeable differences in the distribution of gadolinium between the tumor lines. In half of the U251 animals, Gd-contrast produced characteristic ring enhancement around a dark central region, consistent with the commonly observed structure of a highly-proliferative and well-perfused tumor region surrounding an often necrotic region with inadequate blood supply. In these animals, the Gd-enhanced regions showed elevated fluorescence activity while the tumor “core” showed very low levels of fluorescence yield. None of the 9L-GFP tumors produced this ring enhancement feature, instead showing fairly consistent distribution of the Gd throughout the tumor region. This suggests that the vascular structure was markedly different between the tumor lines and raises the possibility that the results manifested more from differences in uptake and clearance rates than EGFR expression. While the 48-hour delay between the administration of the dye and imaging reduces the potential impact of the EPR kinetics, even at these long time points this factor cannot be dismissed. Further investigations using unbound drugs and competitive binding analyses may help settle this question.

The fluorescence imaging algorithms deployed in this study are based on two-dimensional light propagation models which likely introduced the optical imaging equivalent of partial volume effects. Though only a single plane of optical data was acquired with the measurement system, light propagates well outside of this plane and therefore measurements contain substantial information from regions of the tissue not included in the imaging plane. While expanding the optical measurement system into the third dimension is difficult due to the

limited space in an MRI magnet bore, the light propagation model used in the image recovery algorithms may readily be extended to three dimensions, resulting in a more accurate model of light propagation. Migrating to full volumetric MRI-FMT imaging algorithms, even without changing the optical animal interface, should improve the diagnostic capabilities of the system.

Conclusion

The pre-clinical results presented in this paper suggest that MRI-FMT with fluorescent EGF provides excellent discrimination based on EGFR status. Reliable quantification of receptor status using minimally-invasive techniques would be an important innovation for investigating new and existing cancer treatments that target these cellular mechanisms in research animals. Applying the same principles to identify receptor amplification in human brain cancer patients may provide a less invasive procedure for tailoring treatment regimens and monitoring the tumor molecular expression as it changes through the course of therapy.

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Figure 1.

A study mouse positioned in the MRI rodent coil with eight optical fibers surrounding the head is shown in (a). (b) presents a 3-D rendering of the surface of the mouse head generated from an MRI image stack and illustrates the location of the optical fibers on the tissue (represented by red spheres). A sample from a coronal Gd-MRI image series for a U251 tumor-bearing mouse is shown in (c) through (f). After (e) was segmented into general tissue regions, (g), this information is used to guide the reconstruction of fluorescence yield, shown as an overlay on the corresponding image in (h).

Figure 2.

From top to bottom: Coronal MRI-FMT images of fluorescence yield in the mouse head [(a) and (b)], ex-vivo fluorescence scans of the same animals [(c) and (d)] and corresponding histopathology slides [(e) and (f)]. These representative images are shown for animals with U251 and 9L-GFP tumors in the first and second columns, respectively. The recovered fluorescence activity in the 9L-GFP was very low and thus the outline of the Gd-enhanced region is included for illustrative purposes, (b). The outline in (d) shows the boundary of the brain in the head. Arrows in (e) and (f) point to tumor tissue determined by a surgical pathologist.

(b)

Group comparison	Area under curve (AUC)
Controls (4) vs. U251 (6)	1.0
Controls (4) vs. 9L (5)	0.55
U251 (6) vs. 9L (5)	1.0

Figure 3.

The box and whisker plot in (a) compares the mean values of the recovered fluorescence yield in regions delineated by gadolinium enhancement between the U251 and 9L-GFP mouse populations. Since no gadolinium enhancement was observed in control mice, the mean value of fluorescence yield in the entire brain was used. Area-under-the-curve values determined from ROC analysis of the data in (a) are tabulated in (b).