Folate receptor overexpression can be visualized in real time during pituitary adenoma endoscopic transsphenoidal surgery with near-infrared imaging

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OBJECTIVE Pituitary adenomas account for approximately 10% of intracranial tumors and have an estimated prevalence of 15%–20% in the general US population. Resection is the primary treatment for pituitary adenomas, and the transsphenoidal approach remains the most common. The greatest challenge with pituitary adenomas is that 20% of patients develop tumor recurrence. Current approaches to reduce recurrence, such as intraoperative MRI, are costly, associated with high false-positive rates, and not recommended. Pituitary adenomas are known to overexpress folate receptor alpha (FRα), and it was hypothesized that OTL38, a folate analog conjugated to a near-infrared (NIR) fluorescent dye, could provide real-time intraoperative visual contrast of the tumor versus the surrounding nonneoplastic tissues. The preliminary results of this novel clinical trial are presented.

METHODS Nineteen adult patients who presented with pituitary adenoma were enrolled. Patients were infused with OTL38 2–4 hours prior to surgery. A 4-mm endoscope with both visible and NIR light capabilities was used to visualize the pituitary adenoma and its margins in real time during surgery. The signal-to-background ratio (SBR) was recorded for each tumor and surrounding tissues at various endoscope-to-sella distances. Immunohistochemical analysis was performed to assess the FRα expression levels in all specimens and classify patients as having either high or low FRα expression.

RESULTS Data from 15 patients (4 with null cell adenomas, 1 clinically silent gonadotroph, 1 totally silent somatotroph, 5 with a corticotroph, 3 with somatotrophs, and 1 somatocorticotroph) were analyzed in this preliminary analysis. Four patients were excluded for technical considerations. Intraoperative NIR imaging delineated the main tumors in all 15 patients with an average SBR of 1.9 ± 0.70. The FRα expression level of the adenomas and endoscope-to-sella distance had statistically significant impacts on the fluorescent SBRs. Additional considerations included adenoma functional status and time from OTL38 injection. SBRs were 3.0 ± 0.29 for tumors with high FRα expression (n = 3) and 1.6 ± 0.43 for tumors with low FRα expression (n = 12; p < 0.05). In 3 patients with immunohistochemistry-confirmed FRα overexpression (2 patients with null cell adenoma and 1 patient with clinically silent gonadotroph), intraoperative NIR imaging demonstrated perfect classification of the tumor margins with 100% sensitivity and 100% specificity. In addition, for these 3 patients, intraoperative residual fluorescence predicted postoperative MRI results with perfect concordance.

CONCLUSIONS Pituitary adenomas and their margins can be intraoperatively visualized with the preoperative injection of OTL38, a folate analog conjugated to NIR dye. Tumor-to-background contrast is most pronounced in adenomas that overexpress FRα. Intraoperative SBR at the appropriate endoscope-to-sella distance can predict adenoma FRα expression status in real time. This work suggests that for adenomas with high FRα expression, it may be possible to identify margins and to predict postoperative MRI findings.

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KEY WORDS pituitary surgery; fluorescence; folate receptor; visualization
Pituitary adenomas have an estimated prevalence of 15%–20% in the US, and they account for approximately 10% of intracranial tumors. Although they are predominantly benign, pituitary adenomas can still cause significant disability from compression of the adjacent neural structures and hypersecretory syndromes.

While prolactinomas and some somatotroph adenomas can be medically managed with cabergoline and octreotide, respectively, surgical management remains the primary treatment for nonfunctional (NF) adenomas and other functional adenomas. Currently, the endoscopic transsphenoidal approach is the most common surgical approach for resecting pituitary adenomas. The extent of resection is based on the surgeon’s visual and tactile impression of the tissue. Achieving complete resection can be difficult, and the tumor recurrence rate can be as high as 20% after surgery. Some surgeons have resorted to implementing MRI scanners in the operating room to maximize extent of resection, but this remains expensive and limited in availability. In addition, intraoperative MRI has been shown to result in a high false-positive rate and is not currently recommended. This study proposes a novel optical contrast technique that takes advantage of the pituitary adenoma’s overexpression of folate receptors.

Folate receptor alpha (FRα) overexpression has been reported in pituitary adenomas, especially NF adenomas, which overexpress FRα more than 20 times above the level of the normal pituitary gland and surrounding intracranial structures. This makes FRα an attractive target for specific labeling of pituitary adenoma with a fluorescent dye that can be visualized intraoperatively. OTL38 (On Target Laboratories) is a folic acid analog conjugated to an analog of indocyanine green (ICG), a near-infrared (NIR) fluorescent dye. This conjugated dye binds folate receptors. Fluorescence in the NIR range has advantages over visible light by virtue of its superior tissue penetration and lack of autofluorescence in normal tissues. We hypothesized that OTL38 would selectively bind to the folate receptors on pituitary adenomas that overexpress FRα and allow intraoperative visualization of neoplastic tissue.

Methods

Study Design

This prospective cohort study was approved by the University of Pennsylvania Institutional Review Board, and all patients gave informed consent. Adult patients over the age of 18 years who presented with a pituitary tumor were eligible for this study. Pregnancy and history of allergy to Benadryl (Johnson & Johnson) or OTL38 were the main exclusion criteria. All patients underwent preoperative MRI of the brain and sella with and without gadolinium contrast (Table 1). Patients were informed that enrollment in the study would not substantially change the scope of surgery, as resection would proceed with conventional techniques. Margin biopsies, however, would be obtained to study the value of intraoperative NIR imaging.

OTL38 Administration

Patients who were enrolled in this study were instructed to stop taking any folate supplements, including multivitamin, 48 hours prior to surgery to reduce interactions with OTL38. Two to 4 hours prior to surgery, patients were injected with 25 mg Benadryl to minimize allergic reactions, and 0.025 mg/kg OTL38 was infused over the course of 1 hour. Patients were monitored for adverse reactions during and 30 minutes after infusion, and then they were checked in for surgery.

Surgical Approach

A uninostril or binostril endoscopic endonasal approach was performed in all patients. The endonasal approach was performed by otorhinolaryngologists, primarily using an endoscopic camera and endoscope system (Storz). The choice of the uninostril or binostril approach was made based on patient anatomy. A standard transnasal approach was employed by opening both sphenoid sinuses, removing the sphenoid rostrum, and resecting the sphenoid intersinus septum. The mucosa of the sella was removed in anatraumatic fashion to keep the surgical field dry. After exposing the sella face and sella floor, the sella was opened using standard techniques, and the dura was kept intact as much as possible to visualize NIR signal through the dura. Once the dura was opened, the NIR camera system was used to visualize fluorescence within the tumor. Tumor resection was performed using standard Storz endoscopic equipment, and, on completion, the NIR camera system was brought back in to inspect the resection margins. On satisfactory completion of the surgery, closure was performed jointly by otorhinolaryngology and neurosurgery personnel depending on the need for CSF leak repair. Abdominal fat was harvested only in select cases; in other instances, either a free mucosal graft or nasoseptal flap was used.

NIR System

In all cases, the NIR signal was visualized using the Visionsense Iridium camera system (Visionsense). This system is FDA approved for perfusion imaging in plastic and reconstructive surgery (for example, to assess tissue flap vascularity). The Visionsense Iridium camera system was coupled to a dedicated 4-mm outer-diameter endoscope (Fig. 1). The excitation source is a laser tuned to the NIR range (785 nm), and the sensor was filtered for emissions in the 800- to 835-nm range. The endoscope consists of a 4-mm outer-diameter scope, which features a dual optical path design, allowing the separate and independent use of white light and NIR light. Having separate paths for visible light and NIR images allows very faint fluorescence images to be acquired in the presence of strong white light. Image processing was performed in real time and displayed at 1080-pixel video resolution, with recording at 720 pixels.

DSouza et al. recently compared the Visionsense Iridium system with 5 other commercially available fluorescent visualization systems, including those manufactured by PerkinElmer (Solaris), Quest, Novadaq, and others. They concluded that the Visionsense Iridium system demonstrates the highest sensitivity for NIR fluorescent dye; its sensitivity is in the picomolar range for IRDye 800 (LI-COR Biosciences), which has excitation and emission...
<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age (yrs), Sex</th>
<th>IHC Finding</th>
<th>Preop Biochemical Status*†‡</th>
<th>Clinical Features at Presentation</th>
<th>Preop/Postop Visual Field Deficit</th>
<th>Max Tumor Dimension (mm)</th>
<th>Postop Complications</th>
<th>Hormonal Changes</th>
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<tbody>
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<td>67, F</td>
<td>Negative</td>
<td>Mild hyperprolactinemia, hypogonadotropic hypogonadism</td>
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<td>Rt temporal hemianopia, headaches</td>
<td>Yes/yes</td>
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<td>None</td>
<td>Resolving hypogonadism</td>
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<tr>
<td>16</td>
<td>52, F</td>
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<td>Incidental MRI finding</td>
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<td>Self-limited, mild SIADH</td>
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<td>LH, FSH, rare TSH</td>
<td>Elevated alpha subunit, mild hyperprolactinemia</td>
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<td>54, M</td>
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<td>Resolved hypogonadism &amp; hypercortisolemia w/ persistent ACTH elevation</td>
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<td>Elevated IGF-1</td>
<td>Incidental MRI finding, increased ring size</td>
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<td>GH</td>
<td>Elevated IGF-1</td>
<td>Weight gain, new-onset DM, increased ring size, bitemporal hemianopia</td>
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<td>Transient SIADH</td>
<td>Hypothyroid, adrenal insufficiency</td>
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</tbody>
</table>

ACTH = adrenocorticotrophic hormone; DI = diabetes insipidus; DM = diabetes mellitus; FSH = follicle-stimulating hormone; GH = growth hormone; IGF-1 = insulin-like growth factor-1; IHC = immunohistochemical; LH = luteinizing hormone; SIADH = syndrome of inappropriate antidiuretic hormone secretion; TSH = thyroid-stimulating hormone.

* Mild prolactin elevation defined as < 60 ng/ml.
† Mild cortisol defined as urine free cortisol < 2 times the upper limit of normal.
‡ IGF-1 elevation defined as < 2 times the upper limit of normal.
characteristics that are similar to OTL38 and ICG. In addition to high sensitivity, the Visionsense system features a “smart image processing algorithm to produce a wide dynamic range,” which allows for better comparisons of high and low signal-to-background ratios (SBRs).

Study Procedure

On encountering the tumor, the Visionsense Iridium 4-mm endoscope was used to simultaneously view white light and NIR images (Fig. 2). These 2 images were superimposed on the video output in real time to allow intraoperative analysis of the NIR signal. The NIR signal through the dura was recorded, as was the NIR signal through the pituitary adenoma after the dura was opened. After pituitary exposure, specimens were obtained of the mass and coded by the attending surgeon (J.Y.K.L. or M.S.G.) as consistent with the tumor based on the surgeon’s impression under white light (yes vs no) and NIR fluorescence (yes vs no) and then sent to the pathology laboratory for histopathological diagnosis. Surgery then proceeded in the standard-of-care manner without the use of NIR fluorescence.

After the tumor had been resected and the attending neurosurgeon was satisfied that complete resection had been achieved based on white light visualization, NIR imaging was used to identify areas of residual disease (Fig. 2E and F). Areas were biopsied at the discretion of the senior surgeon (J.Y.K.L. or M.S.G.), and specimens were coded and sent to the pathology laboratory.

Patients were admitted to the intensive care unit following surgery, and no adverse outcomes were reported. Postoperative MRI was performed on postoperative Day 1, and the patients were seen approximately 2 and 4 weeks after resection.
Immunohistochemistry for FRα

The specimens obtained from each patient were chemically fixed, embedded in paraffin blocks, and cut to 5-μm-thick sections. These permanent sections were stained with H & E. Furthermore, immunohistochemical analysis against FRα was performed using murine monoclonal antibodies against the folate receptor (1:20 dilution of NCL-L-FRα, Leica Biosystems). To quantify FRα expression in each sample, H-scores were calculated by a single, blinded neuropathologist (M.M.L.) using a positive control as the standard, by summing the percentages of adenoma cells that stained strongly (3+) multiplied by 3, the percentage of adenoma cells that stained moderately (2+) multiplied by 2, and the percentage of adenoma cells that stained weakly (1+) multiplied by 1 (Fig. 3 and Table 2). The H-score ranges from 0 to 300, with 300 implying strong staining in all cells.

NIR Image Analysis of SBR and Laser Distance

The data from 15 patients were analyzed using Vision-sense software (VSPlayer v1.8.05.01; Fig. 2I). Between 3 and 5 region of interest points were placed on the adenoma, and then an additional 3–5 points of interest were placed on the surrounding normal tissue (such as the bone of the tuberculum sella or carotid prominences) to determine the signal-to-background ratio (SBR). Because the intravenous administration of OTL38 causes normal mucosa and skin to fluoresce when the laser source is sufficiently close, fluorescence in the mucosa that was more proximal to the laser than the pituitary mass was disregarded. In general, the surgeon removed the mucosa from the sella and surrounding sella as part of the normal surgical procedure.

Because the Vision-sense endoscope does not have the capacity to measure the distance from the laser source to the fluorescent object, a substitute measurement for estimating this distance was developed. The medial optico-cortid recess (MOCR) provides a useful landmark in the endoscopic approach to the sella.15,17,38 The distance between the 2 MOCRs as a fraction of the overall field size gives a rough estimate of the distance of the laser source to the fluorescent tissue (Fig. 2I).

Data Analysis

Stata (version 10, StataCorp) was used to compute the summary statistics and perform the t-tests and linear regression analysis.

Results

Clinical Data

A total of 19 patients were enrolled between October 2015 and May 2016. Two patients were excluded because the laser serving as the excitation light source malfunctioned during surgery. Two patients were excluded because their final pathological review did not demonstrate pituitary adenoma (craniopharyngioma and Rathke cleft cyst). One patient, who presented with a somatotroph adenoma and tuberculum sellae meningioma, is included in the data analysis, but SBR data were censored due to
interference with the NIR signal measurement from the meningioma.

Data from 15 patients were available for analysis. Seven patients were male, and the mean patient age was 53.2 years (range 35–80 years). Six patients had NF adenomas (4 patients had a null cell adenoma, 1 patient had a clinically silent gonadotroph adenoma, and 1 patient had a totally silent somatotroph adenoma), 3 patients had somatotroph adenomas, 5 patients had corticotroph adenomas, and 1 patient had both corticotroph and somatotroph cells, as determined by immunohistochemical analysis (Table 1).

All patients tolerated injections of 0.025 mg/kg OTL38 administered 2 to 4 hours prior to surgery without adverse events. All patients did well after surgery with no unique complications identified. Seven of 9 patients with functional adenomas had postoperative control of hormonal hypersecretion. A surgical cure was not attained in the remaining 2 patients and further medical treatment was required (Table 1). The rate of gross-total resection (GTR) based on postoperative MRI was 73% (Table 3). Residual tumor was identified on MRI only in patients with cavernous sinus invasion or a significant extrasellar tumor, such as a tumor in the sylvian fissure.

**Intraoperative NIR Fluorescence Using the Visionsense System**

All pituitary adenomas demonstrated some degree of a NIR fluorescent signal compared with background. Taken as a whole, the mean ± SD SBR was 1.9 ± 0.70 for all samples in all 15 patients. There was significant variability in the range of SBR (1–3.9; interquartile range 1.6–2.7). To explain this wide range in fluorescence, we performed a subgroup analyses using several variables: functional status (known preoperatively), distance from the endoscope to the pituitary gland (determined using the distance between the MOCRs as a proxy and known at time of surgery), time from injection (known at time of surgery), and FRα expression level (not known until after surgery was completed).

**FRα Overexpression**

Previous studies have demonstrated that NF pituitary adenomas overexpress FRα compared with functioning pituitary adenomas. Immunohistochemical analysis was performed on paraffin-embedded tissue sections using murine monoclonal antibodies to FRα, sections were examined by a single neuropathologist (M.M.L.), and H-scores (0–300) were determined (Fig. 3 and Table 2).

Three of the 6 NF adenomas demonstrated significant FRα overexpression with H-scores of 200, 225, and 270. These tumors were categorized as "high FRα tumors." Among the 3 other patients with NF adenomas, 1 patient had a totally silent but growth hormone–staining adenoma. This tumor demonstrated an H-score of 100. In comparison, normal adenohypophysis has an H-score of 90 based on specimens that contain some small amount of normal pituitary gland tissue in the tissue block. Because 1 patient with an H-score of 100 was not significantly above the background H-score of the normal pituitary gland, we

### TABLE 2. Results of immunohistochemical analysis of FRα

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<tr>
<th>Case No.</th>
<th>Pathology</th>
<th>FRα Staining Intensity</th>
<th>% Cells Stained</th>
<th>H-Score</th>
<th>SBR Close (MOCR &gt;0.7)</th>
<th>SBR Middle (MOCR 0.4–0.7)</th>
<th>SBR Far (MOCR &lt;0.4)</th>
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NA = not applicable (images were not taken at this distance).
* Patient presented with an adenoma and tuberculum sellae meningioma.
chose to categorize this patient as having a “low FRα tumor.” The other 2 NF adenomas were null cell adenomas and demonstrated virtually no FRα overexpression.

Among the 5 patients with corticotroph adenomas, all demonstrated low FRα expression, with H-scores ranging from 0 to 10. The 3 patients with somatotroph adenomas had H-scores ranging from 0 to 5, and 1 patient with somatocorticotroph adenoma had an H-score of 40.

Overall, only 3 patients were considered to have high FRα overexpression, and 12 patients were considered to have low FRα expression.

The NIR SBR of the patients with low H-scores was 1.6 ± 0.43, and the NIR SBR of the patients with high H-scores was 3.0 ± 0.27 (p < 0.01, t-test). Thus, the 3 patients with NF adenoma and high FRα overexpression (defined as > 200) did show significantly higher NIR SBR compared with the other 12 patients.

**Functional Status**

Six of the 15 patients had NF adenomas. One patient’s tumor positively stained for gonadotroph hormone on immunohistochemistry and 1 patient’s tumor positively stained for somatotroph hormone, but the preoperative laboratory values were not suggestive of hormonal abnormality. Because clinical hormonal status is the primary information that would be known at the time of resection, rather than pathological hormone staining, we plotted SBR versus functional status. The NIR SBR of NF adenomas (2.4 ± 0.81) was higher than the NIR SBR of functional adenomas (1.6 ± 0.48) (p < 0.05). However, because mean values are sensitive to extreme values (e.g., tumors with an FRα H-score > 200), we interpreted the data with a box plot to better evaluate the data (Fig. 4). As can be seen in Fig. 4, there is a significant overlap in the SBRs of functional and nonfunctional tumors.
NF adenomas: the functional status of the adenoma alone did not explain the variability in SBR.

**Endoscope Distance**

The signal intensity of light varies by the square of the distance, and as such we hypothesized that the endoscope distance to the sella may contribute to variability in SBR, especially within the tight confines of the nose and sella. In the absence of a reference standard, we measured the MOCR distance on the screen and divided it by the horizontal width of the video itself, which is captured as 720 line pairs in the horizontal dimension: MOCR ratio = (medial opticocarotid recess distance)/(horizontal video capture distance).

The MOCR fraction ranged from 0.33 (which is one-third of the screen, implying that the endoscope is relatively far from the sella) to 1.0 (which means the MOCR is filling the entire sella). Indeed, the MOCR fraction can actually be > 1.0 if the endoscope is introduced into the sella itself; however, we limited calculations of SBR to the more typical distances at which the sella is visualized.

MOCR was not a statistically significant univariate predictor of SBR in all patients (linear regression, p = 0.59). However, multivariate analysis, which takes FRα expression into consideration in the linear regression model, did identify statistical significance. In both adenomas overexpressing FRα and adenomas that do not, the endoscope distance from the adenoma influenced SBR (Figs. 5–7).

**Time From Injection to Visualization**

Although the time required for OTL38 to saturate a tumor is proposed to be less than 30 minutes, its half-life in circulation is only approximately 25 minutes, suggesting that clearance of OTL38 from receptor-negative tissues...
cuss this issue in the limitations section in Discussion time from injection increased (p = 0.0274; Fig. 8). We dis-
signal, we identified a linear trend toward higher SBR as
should theoretically not demonstrate any significant NIR
the rapid uptake of OTL38 by FR
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nificant time dependence for SBR, which is consistent with
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negative tissues. For the 3 patients with FR
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overexpres-
sualization using endoscopic and microscopic transsphe-
 should require 3–5 hours (i.e., 5 half-lives of OTL38 clear-
ance should reduce the level of unbound imaging agent in
circulation to < 5% of its initial level and the correspond-
ing concentration of OTL38 in the tissues should soon fol-
low). Because the fraction of OTL38 that is captured by a
folate receptor will likely be internalized and retained by
receptor-expressing cells, NF adenomas that express high
levels of FRα should appear brightly fluorescent within
approximately 30 minutes of injection and maintain their
bright fluorescence for hours or even days.
In this study, the time from intravenous injection to ini-
tial surgical visualization of the adenoma ranged from 166
minutes to as long as 390 minutes, allowing sufficient time
for most of the unbound OTL38 to clear from receptor-
negative tissues. For the 3 patients with FRα overexpres-
sion (regression p value = 0.28) and 5 patients with NF
adenomas (regression p value = 0.5), we did not find a sig-
ificant time dependence for SBR, which is consistent with
the rapid uptake of OTL38 by FRα-overexpressing cells.
However, in patients with low FRα expression, who
should theoretically not demonstrate any significant NIR
signal, we identified a linear trend toward higher SBR as
time from injection increased (p = 0.0274; Fig. 8). We dis-
cuss this issue in the limitations section in Discussion.

NIR as a Predictor of Postoperative MRI Results
All patients underwent postoperative MRI within 48
hours of surgery. Four of 15 patients showed residual neop-
plasm (Table 3). Excluding the patient in whom the laser
malfunctioned in the middle of the procedure, 14 of 15
patients had intraoperative NIR images that were analyzed
for fluorescence after excision of the main tumor. One pa-
tient demonstrated definite residual fluorescence and neop-
plasm on MRI, which was expected intraoperatively given
the intracranial lateral extent of the neoplasm (Table 3 and
Fig. 9). Her second-stage craniotomy performed 96 hours
later demonstrated strong fluorescence even 4 days after
injection, which is consistent with the anticipated internal-
ization of the dye by the FRα-overexpressing adenoma
cells. Two of 3 patients with cavernous sinus involvement
seen on preoperative MRI exhibited residual neoplasm in
the cavernous sinus or adjacent to the cavernous sinus on
postoperative Day 1. In one of these patients (Case 12), the
residual neoplasm was not seen using visible light alone,
but a residual NIR signal was identified, thus predicting
the findings on the follow-up MRI scan obtained 1 month
later (Fig. 5).
For the 3 patients with high FRα expression, the post-
operative MRI results correlated perfectly with the intra-
operative imaging findings, and the residual fluorescence
seen in 2 patients corresponded to the residual neoplasm
seen on postoperative MRI (Table 3).

Using NIR to Detect Margins
On completion of standard adenoma resection, the Vi-
sionsense Iridium endoscope was introduced to inspect
the margins of any potential residual adenoma. Excluding
4 patients from whom margin specimens were not taken,
a total of 16 margin samples were collected from 11 pa-
tients. For all specimens, the true-positive rate was 86%
and the true-negative rate was 89% using NIR, while the
true-positive and true-negative rates were 80% and 89%,
respectively, using visible light. If we exclude functioning
adenomas and only examine the 6 patients with NF ade-
nomas, a total of 5 margin samples were collected; for both
NIR and visible light, the true-positive rate was 100% and
the true-negative rate was 100%. Focusing on the 3 patients
with FRα overexpression, a total of 3 margin samples were
obtained. Two samples were positive for tumor, and these
same 2 samples were also positive for fluorescence. One
margin specimen was negative for fluorescence, and the
final pathological analysis yielded only “fragments of
crushed normal adenohypophysis.” Hence, the sensitivity,
specificity, positive predictive value, and negative predic-
tive value were 100% for these 3 patients.

Discussion
Despite advancements in the surgical approach and vi-

NIR imaging of FRα for pituitary adenomas

J Neurosurg Volume 129 • August 2018

that although iMRI showed residual tumor, residual pituitary adenomas decreased in volume by an average of 90% in half of these patients on follow-up imaging. Hence, iMRI may result in false positives, and a recent guideline statement from the Congress of Neurological Surgeons concludes that iMRI is not currently recommended. Consequently, adjunct imaging is still needed during pituitary adenoma resection to help achieve GTR.

Pioneering work performed by Evans et al. demonstrated that FRα—a membrane receptor that transports reduced folate—is overexpressed in NF pituitary adenoma cells. Folate is an essential vitamin necessary for nucleic acid biosynthesis and is transported across the cell membrane in 3 ways: ubiquitously expressed reduced folate carrier, intestinal brush border–specific proton-coupled folate transporter, and folate receptor. Folate receptors exist in 4 forms—FRα, FRβ, FRγ, and FRδ—and FRα has been reported to be overexpressed in ovarian, lung, and breast carcinomas. In vivo SPECT imaging of folate receptor binding in healthy volunteers and patients has been performed using 99mTc-labeled etarfolatide, a folate-targeted technetium chelating agent, to demonstrate that folate receptors can be targeted for visualization.

The ability to conjugate a folate molecule to a fluorescent contrast agent has been previously proposed to identify tumor nodules. Specifically, van Dam et al. published the first trial in humans that demonstrated intraoperative optical contrast during surgery for ovarian cancer using a folate-fluorescein (EC17) conjugate to detect intraoperative metastasis. Subsequently, Hoogstins et al. studied the use of OTL38 in ovarian carcinomas, which overexpress FRα. Based on these preliminary findings, we hypothesized that FRα overexpression in pituitary adenomas may provide a window of opportunity for neurosurgeons to visualize pituitary adenomas during surgery. Ultimately, we propose that this technology may be able to improve the GTR of pituitary adenomas.

NIR Visualization

In this study, we investigated the potential of using intraoperative, fluorescent, folate receptor targeting to improve the GTR of pituitary adenoma. For several reasons, we chose to use OTL38 (the NIR fluorescent, folate-conjugated dye) over EC17 (the visible light, fluorescent, folate-conjugated dye as used in the ovarian cancer trial published by van Dam et al.). First, there is minimal autofluorescence in the NIR spectrum in CNS tissues. In addition, NIR has a longer wavelength, which can potentially provide a benefit because NIR light can penetrate the normal dura. In our prior work using Second Window ICG, where we deliver an extremely high dose of ICG intravenously and then wait 24 hours before surgical visualization, we have been able to demonstrate the NIR signal through normal dura and normal brain parenchyma. Another benefit of NIR is that our group has extensive experience using NIR fluorescence to study gliomas and meningiomas. One of the main challenges of working in the NIR spectrum is that humans cannot see light in the NIR spectrum. Indeed, most neurosurgeons have experience using NIR during vascular neurosurgery (for example, aneurysm clipping), and the image is only seen on the screen in black and white. In contrast, we chose to employ the Vi-
sionsense Iridium system, which simultaneously provides the real-time overlay of visible light and NIR as a “pseudocolor” map of the NIR signal superimposed on the visible light view (Figs. 1 and 2). This allowed the surgeon to work with the endoscope in visible light and then turn on NIR when necessary. The Visionsense Iridium system has been shown to have high sensitivity and a wide dynamic range, making it very suitable for our purposes.

**FRα as a Target for Optical Contrast**

In this study, 3 of 6 patients (50%) with NF adenomas demonstrated FRα overexpression based on the immunohistochemical analysis of the paraffin-embedded specimens. The NIR SBR of the 12 adenomas with low FRα was 1.6 ± 0.43, and the SBR of the 3 adenomas with high FRα was 3.0 ± 0.27 (p < 0.01, t-test). Thus, by keeping the endoscope farther from the sella (MOCR < 50% of video screen) and using an SBR cutoff value of 2.5, we could perfectly predict FRα expression in all patients with an area under the receiver operating characteristic curve of 1.0 (Fig. 10). This could be used to predict adenomas that intraoperatively overexpress FRα.

An important finding of this pilot study is that the endoscope distance and injection-to-visualization time are critical. SBR can be artificially increased by simply bringing the endoscope closer to the tumor. We have found that maintaining an endoscope distance that is farther from the sella (where the MOCR spans less than half of the video screen) leads to the best ability to differentiate high and low SBR, which correspond to high and low FRα expression, respectively. There is obvious patient variability with respect to the distance between the right and left MOCRs, as reported by Nunes et al. (the average MOCR distance was 11 ± 1 mm). Nevertheless, in the absence of a laser range finder to provide the absolute distance, we used this internal landmark as a proxy for distance.

**Clinical Benefit**

GTR of an NF pituitary adenoma is often limited by intrinsic factors such as the circumferential invasion of the cavernous sinus (Knosp Grade 4 invasion). Berkmann et al. demonstrated that cytoreduction could be improved in the iMRI suite by taking the time to obtain an MRI scan and then performing further resection. Similarly, real-time intraoperative molecular imaging with an optical contrast agent such as folate-NIR dye may be able to replicate the iMRI findings in the subset of pituitary adenomas that overexpress FRα. We believe that intraoperative, folate receptor–targeted, fluorescent tumor imaging may provide important intraoperative information that influences surgical decision making and allows the surgeon to improve resection. In this limited series of 3 patients with...
NIR imaging of FRα for pituitary adenomas

J Neurosurg Volume 129 • August 2018

high FRα overexpression, the intraoperative fluorescence perfectly predicted the postoperative MRI findings. In 1 patient (Case 7), no residual fluorescence was seen intraoperatively, and no residual tumor was seen on postoperative MRI (Fig. 2). In 2 patients (Cases 1 and 12), intraoperative fluorescence demonstrated a residual adenoma to the left of the sella, and in both cases MRI demonstrated residual adenoma on the left side (Figs. 5 and 10). This suggests a strong correlation between intraoperative fluorescence imaging and postoperative MRI.

With respect to margin detection, we believe that this technique has excellent sensitivity and specificity for patients with high FRα expression. In a margin analysis of 3 patients with high FRα expression, OTL38 showed major clinical value. Three biopsy specimens were obtained from 2 of the patients. There was a perfect correlation between intraoperative NIR fluorescence and the pathological analysis, demonstrating 100% sensitivity, specificity, positive predictive value, and negative predictive value. We recognize, of course, that these results must be extended to a larger population of patients with NF pituitary adenoma.

Limitations

A shortcoming of our study was the small number of patients who had FRα-overexpressing adenomas. Evans et al. concluded that all 11 NF adenomas demonstrated positive FRα overexpression on immunohistochemical analysis, and none of 7 functioning adenomas demonstrated this overexpression.7 In our study, none of 9 functioning adenomas demonstrated FRα overexpression, and only 3 of 6 (50%) of the NF adenomas demonstrated FRα overexpression. Because of this low FRα overexpression, we primarily focused this pilot study on predicting FRα overexpression using pathological immunohistochemical analysis as the gold standard. Without the proper identification of those tumors with high FRα overexpression, this technique could result in significant false-negative results. We will continue to expand this work in nonfunctioning adenomas, but there may also be a role for predicting which patients will benefit from this dye before surgery. Preoperative radionuclide imaging with 99m Tc EC20 could be a viable option.19,32,37

Another limitation of this study is that we were only able to analyze 5 margin specimens of adenomas with an FRα H-score > 100 in order to compare visual and tangible discrimination of the tumor to OTL38 fluorescence, which showed that both approaches correctly discriminate neoplasms from normal tissue with 100% sensitivity and specificity. Future studies with larger sample sizes will further examine the validity of using OTL38 for margin discrimination.

A third limitation of the study is the possibility of false positives when using the NIR signal. Many pituitary adenomas in this study did not overexpress FRα and thus should not have demonstrated any significant NIR signal. Nevertheless, we believe that there may be a small accumulation of OTL38, even in the pituitary adenomas as shown in Figs. 6 and 8. This observation likely derives from the fact that the tumor vasculature of a pituitary adenoma is generally leakier than healthy tissue vasculature, leading to an enhanced permeation and retention effect in the tumor that allows some contrast agents to passively accumulate in the tumor tissue more effectively than in normal tissues.39 Because the magnitude of this effect is small, especially with low-molecular-weight contrast agents such as OTL38, significant contrast cannot be seen until the agent is cleared from normal tissues. Moreover, because the contrast agent is not receptor bound, it will wash out of the tumor over time. We suspect that adenomas with little or no FRα expression show increased SBR as time passes because the normal tissues clear OTL38 faster than the tumor. Thus, if OTL38’s injection-to-visualization time exceeds 5–6 hours, adenomas with low FRα expression may demonstrate high SBR, leading to false-positive findings.

Another consideration is that there are other folate receptor variants, such as FRβ, that are expressed on activated, but not quiescent, macrophages. We did not stain for FRβ in this study because we did not see macrophage accumulation in the slide blocks. Nevertheless, this is a potential source of OTL38 uptake that would result in fluorescence.

A final issue that needs to be considered is that there is a learning curve to intraoperatively performing video analysis of the NIR signal using the Visionsense Iridium 4-mm endoscope. Although the endoscope has a flat field of illumination in the visible light spectrum, the NIR excitation light source is brighter in the center than in the periphery. Thus, background points must be taken closer to the tumor rather than at the peripheries of the field. In addition, in the absence of a laser range finder to measure distance, approximating MOCR and screen size is not always easy if the surgeon has not opened the sphenoid sinus wide enough to visualize these anatomical structures.

Conclusions

In this paper, we present the first clinical study utilizing a folate receptor–targeted fluorescent dye for resection of pituitary adenomas. Administering the intravenous folate dye, OTL38, safely provided the optical contrast that allowed the discrimination of high and low FRα expression. We confirmed the findings of previous studies that reported only NF pituitary adenomas overexpress FRα. By just focusing on the 3 patients with confirmed FRα overexpression, OTL38 was able to predict postoperative MRI findings and provide 100% sensitivity and specificity of the margins. Although these results are encouraging, we consider this paper to represent an early-stage innovation.

In our future work, we will focus only on NF adenomas in which this modality is the most likely to be clinically relevant. In addition, we will continue to refine the imaging technique and analysis as distance appears to be a critical factor.

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Disclosures
The authors report the following. Dr. Lee owns stock options in Visionsense. Dr. Low has direct stock ownership in On Target Laboratories. Dr. Singhal holds patent rights over the technologies presented in this article.

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