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Feasibility of folate receptor-targeted intraoperative fluorescence imaging during staging procedures for early ovarian cancer

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Summary

Objectives: Completeness of staging is an independent prognostic factor for survival in surgical staging procedures for early ovarian cancer. Near-infrared (NIR) fluorescence imaging has the potential to improve the intraoperative assessment of metastatic spread and thus completeness of staging. Feasibility of folate receptor alpha (FRα) targeted fluorescence imaging using OTL-38, a folate analogue conjugated to an NIR fluorescent dye, has been previously demonstrated in advanced ovarian cancer. The present authors hypothesized that in early ovarian cancer, fluorescence imaging using OTL-38 could lead to more accurate detection of (occult) ovarian cancer metastases, allowing gynecologic surgeons to take targeted rather than blind biopsy samples. *Materials and Methods:* Six patients scheduled to undergo a staging procedure for suspected early stage ovarian cancer, received an intravenous infusion of 0.0125 mg/kg OTL38 2-3 hours prior to surgery. The authors assessed tolerability, pharmacokinetics, and the feasibility of intraoperative NIR fluorescence detection of ovarian cancer lesions. Feasibility was evaluated using histopathological analysis, tumor-to-background ratio, and number of false positive and negative lesions. *Results:* Distinction between a malignant and benign primary tumor was possible with OTL-38 based fluorescence imaging. In addition, nine fluorescent lesions, all lymph node (LN) clusters, were detected intraoperatively. Tumor cells were not demonstrated in any of the biopsy samples taken during staging procedures, including the fluorescent lesions. Therefore all fluorescent LNs were false positives. *Conclusions:* Metastatic lesions were not present in the patients with confirmed early ovarian cancer; hence the anticipated added value of NIR fluorescence imaging could not be demonstrated in this study. Fluorescence imaging led to resection of non-malignant LNs, as comprehensive lymph node dissection should be pursued in surgical staging procedures, this should not impede application of OTL38. Importantly, fluorescence imaging allowed distinction between a malignant and benign primary tumor and had no false negatives.

Key words: Early-stage ovarian carcinoma; Surgical staging; Image-guided surgery; Fluorescence; Lymph node metastasis.

Introduction

In ovarian cancer, distinction is made between early and advanced stage disease. Both surgical procedure and the need for (neo) adjuvant treatment differ between stages. In advanced ovarian cancer (FIGO IIb-IV), a cytoreductive procedure is performed. During this procedure complete cytoreduction of all cancer lesions is the primary goal, as the amount of residual tumor negatively impacts survival [1, 2]. In early ovarian cancer (FIGO I-IIa), a surgical staging procedure is performed. During surgical staging biopsy, samples of clinically suspected areas are obtained. These are supplemented with cytology of the abdominal fluid and biopsy samples of predefined areas, which are typical locations of ovarian cancer metastases. These include pelvic and para-aortic lymph nodes (LNs), right hemi-diaphragm, paracolic gutters, pelvic sidewalls, ovarian fossa, bladder peritoneum, and recto-uterine pouch. The purpose of these ' blind biopsies' is to determine whether there is occult microscopic metastatic spread. In case of metastatic spread, patients are upstaged and thus require additional treatment, i.e. chemotherapy. When the ovarian cancer has not metastasized and is true early stage, resection of the primary tumor is adequate and chemotherapy can be omitted [3-5]. Consequently, clear intraoperative assessment of the presence of metastatic spread is of utmost importance during staging procedures.

Near-infrared (NIR) fluorescence imaging is a relatively novel imaging modality, which makes use of invisible fluorescent light to enhance contrast between target and background tissue. Various favorable optical properties, i.e. fast acquisition time, low autofluorescence, and penetration depths up to 1 cm, make NIR fluorescence imaging eminently suitable for intraoperative application [6-8]. In oncologic surgery, NIR fluorescence imaging can enable surgeons to clearly distinguish malignant from benign tissue [9, 10]. This commonly requires the administration of

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an exogenous fluorescent contrast agent. Preferably these agents specifically target biomarkers that are overexpressed on tumor cells.

Folate receptor alpha (FRα) is a biomarker is strongly expressed on > 90% of epithelial ovarian cancers, while expression on healthy tissues is low [11-13]. This prompted the development of FRα targeting agents for fluorescence imaging of ovarian cancer. These agents, EC17 (fluorescence outside the NIR spectrum) and OTL38 (fluorescence within the NIR spectrum), were studied in ovarian cancer patients in previous clinical trials [14-16]. Feasibility of FRα targeted fluorescence imaging was demonstrated, as the use of these agents allowed gynecologic surgeons to visualize and resect more ovarian cancer lesions. Regrettably the use of OTL38 also had a drawback as in 23% of resected fluorescent lesions, histopathology could not confirm the presence of tumor cells [15]. This false positive fluorescence was mainly seen in lymph nodes (LNs). Activated macrophages in the sinuses of LNs express folate receptor beta (FRβ), which also appeared to be a target for OTL38 [17-19]. Nevertheless, in advanced ovarian cancer the use of intraoperative NIR fluorescence imaging led to better visualization of cancer lesions and consequent resection of 29% additional lesions that were not detected with visual inspection. The present authors hypothesized that application of NIR fluorescence imaging in early ovarian cancer could lead to more accurate detection of (occult) ovarian cancer metastases and could allow gynecologic surgeons to take targeted rather than blind biopsy samples during surgical staging. Completeness of surgical staging is an independent prognostic factor for overall survival, aiming to discriminate true early ovarian cancer from advanced ovarian cancer. This study could provide a significant step towards improving surgical staging and consequently tailored treatment in early stage ovarian cancer patients.

Materials and Methods

OTL38 (chemical formula: C61H63N9Na4O17S4; molecular weight: 1414.42 Da) consists of a folate analogue conjugated to an NIR fluorescent dye. OTL38 (> 96% purity) was obtained. The drug was synthesized and manufactured in compliance with Good Manufacturing Practices. OTL38 was stored in frozen form at −20°C in vials containing 6 mg OTL38 free acid in 3 mL water. Before administration, the frozen vials were thawed, vortexed, and then diluted in 220 mL 5% dextrose for intravenous infusion. Patients received a one-hour intravenous infusion of 0.0125 mg/kg OTL38 2-3 hours before the start of surgery. This dose and the time interval between dosing and surgery were deemed optimal in a previous study[15],

The objectives of the study were to assess tolerability, pharmacokinetics (PK), and the feasibility of intraoperative NIR fluorescence detection of ovarian cancer lesions using a single intravenous dose of OTL38. Tolerability assessment (blood pressure, pulse, peripheral oxygen saturation, respiratory rate, ECG, temperature, and skin assessments), blood collection for PK, and routine laboratory tests were performed at fixed time points starting shortly before administration and lasted up to 24 hours after dosing. Adverse events and the concomitant use of other medications were recorded. Feasibility was assessed by measuring the following endpoints: tumor-to-background ratio (ratio between fluorescent signal of the tumor and fluorescent signal of the background), co-localization of tumor cells on hematoxylin & eosin (H&E), and FRα staining on immunohistochemistry (IHC) with fluorescence; the number of additional cancerous lesions detected with NIR fluorescence imaging and number of false positive and negative lesions. As this study was exploratory in nature, the sample size was not based on statistical considerations.

The study was approved by a certified medical ethics review board (Medical Ethics Committee of Leiden University Medical Center [LUMC]) and was performed in accordance with the laws and regulations on drug research in humans in the Netherlands. All patients provided written informed consent prior to the start of any study-related procedures. The study was registered in the European Clinical Trials Database under numbers 2014-002352-12; publicly accessible via the CCMO register (https://www.toetsingonline.nl/to/ccmo_ search.nsf/Searchform?OpenForm).

The authors included six patients who had a clinical suspicion of early stage epithelial ovarian cancer and were scheduled for a laparoscopic or open surgical staging procedure. Potential patients were selected from the multidisciplinary consent meeting of the LUMC Department of Gynecology between December 2015 and July 2016. The main exclusion criteria were current pregnancy, history of anaphylactic reactions, impaired renal function (defined as eGFR < 50 mL/min/1.73 m^2), and impaired liver function (defined as alanine aminotransferase, aspartate aminotransferase, or total bilirubin levels that exceeded three times the established upper limit of normal).

Imaging was performed using an NIR fluorescence imaging system [20]. Both systems consist of three wavelength-isolated light sources, including a “white” light source and two separate NIR light sources. Color video and fluorescence images were acquired simultaneously using separate sensors and were displayed in real time using custom-built optics and software, thereby displaying color video and NIR fluorescence images separately. A pseudo-colored (lime green) merged image of the color video and fluorescence images was also generated. During open surgery, the camera and moveable arm were enclosed in a sterile shield and drape. During laparoscopic surgery, a sterilized laparoscope and light cable were used.

Surgical staging, as described in the introduction, were open or laparoscopic procedures performed by an experienced gynecologic oncologist. First, suspected lesions were identified in the surgical field using standard visual and, in case of open surgery, tactile methods. Thereafter, the Quest imaging system was used to identify NIR-fluorescent lesions. All suspect lesions identified by visual/tactile methods and NIR fluorescence were resected, when surgically feasible. All resected suspect lesions and biopsy samples, were marked on a case report form as being either fluorescent or non-fluorescent and as being either clinically suspected of malignancy or not.

An experienced pathologist examined all resected lesions for tumor status. A tumor-positive lesion that was fluorescent was considered a true positive, a tumor negative lesion that was fluorescent was considered a false positive, and a tumor-positive lesion that was non-fluorescent was considered a false negative. To assess the origin and relative strength of fluorescence signal, formalin-fixed, paraffin-embedded (FFPE) samples were assessed

Table 1. — *Characteristics of the surgical procedures and tumor histopathology.*

ID	Primary tumor in situ	Diagnosis (stage)	Surgical procedure	Surgical procedure result	Intraoperative fluorescence	
1	No	Endometriod adenocarcinoma (IA)	Laparotomy	Complete staging	Yes	False positive LNs
2	Yes	Mucinous cystadenoma (n.a.)	Laparotomy	Resection primary tumor	No	Concordant with benign tumor
3	No	Mucinous borderline tumor (IA)	Laparoscopy	Complete staging	No	Concordant with absence of metastases
4	Yes	Endometriod adenocarcinoma (IIIA)	Laparotomy	Complete primary debulking	Yes	Concordant with malignant tumor
5	No	Serous adenocarcinoma (IA)	Laparoscopy	Complete staging	Yes	False positive LNs
6	No	Serous adenocarcinoma (IA)	Laparoscopy	Incomplete staging	Yes	False positive LNs

using an imager. In addition, the authors performed immunohistochemistry (IHC) to demonstrate FRα, FRβ, and CD68 (a pan macrophage marker) expression in FFPE sections. Lastly a series of six successive sections were stained alternately with H&E and cytokeratin, in accordance with sentinel LN ultra-staging protocol. For assessment of fluorescent signal arising from OTL38 in sections, a flatbed scanner was used.

Bioanalysis was performed using validated methodologies in compliance with good clinical laboratory practices at Analytical Biochemical Laboratory. In brief, OTL38 was extracted from human K2EDTA plasma samples using off-line solid-phase extraction, followed by analysis using liquid chromatography/mass spectrometry. The assay's lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) were 2.00 and 500 ng/mL, respectively. The coefficient of variability for intra-day and inter-day plasma LLOQ was 8.2%.

SPSS statistical software package (version 23.0) was used for statistical analyses. Patient characteristics are reported as the median, SD, and range. The fluorescence signal in the tumor and background tissue was quantified using ImageJ (version 1.49b, NIH, Bethesda, MD; http://imagej.nih.gov/ij/). Using ImageJ, a region of interest (ROI) was drawn on the images and used to quantify the fluorescence signal in arbitrary units (AU). Tumor-to-background ratio (TBR) was calculated by dividing the fluorescence signal of the tumor by the fluorescence signal of the surrounding healthy tissue. To compare the TBR values and fluorescence background signals between malignant and benign (i.e., false-positive) lesions, and between different dose groups, an independent samples Student's *t*-test was performed. TBR was reported as the mean, SD, and range. The individual OTL38 PK profiles were analyzed using non-compartmental methods.

Results

Six patients, with a mean age of 58 (SD 8.2, range 43-66) years with a clinical suspicion of early ovarian cancer were included. Characteristics of the surgical procedures and tumor histopathology are summarized in Table 1. In four patients a laparoscopic staging was initiated, in one patient the laparoscopy was converted to a laparotomy because of massive adhesions. The remaining two patients underwent an open procedure using a midline abdominal incision. In four patients, the primary tumor, i.e. adnexa, had already been resected during a prior procedure, in the other patients the primary tumor was still in situ. Following intraoperative frozen section analysis, the staging procedure was abandoned in two patients. In one patient the staging was halted after the primary tumor was found to be benign. In the other patient the staging was converted to a debulking procedure as a consequence of macroscopic gross disease outside the pelvis. In the remaining four patients a staging procedure was performed, in one patient staging was incomplete as biopsy samples from right paracolic gutter and the right hemi-diaphragm were erroneously forgotten.

In two out of six patients symptoms suggestive of hypersensitivity (e.g., dysphonia and pruritus) occurred during OTL38 infusion. These symptoms were mild in severity and self-limiting. No serious adverse events or deaths related to OTL38 occurred during the study period. Administration of OTL38 did not lead to any apparent changes in laboratory values, ECG, vital signs, or temperature.

The maximum blood plasma concentration of OTL38 was achieved at the end of the infusion and subsequently declined with an elimination half-life of two to three hours, similar to earlier results[15].

In two patients the primary tumor, i.e. adnexa, was still in situ; in one patient the tumor was fluorescent (TBR 4.5) while in the other patient fluorescence was not detected (Figures 1A-B). The fluorescent tumor was subsequently confirmed as an ovarian malignancy on frozen section, while the non-fluorescent tumor proved to be a benign mucinous cysteadenoma.

In three out of the four patients who underwent a staging procedure fluorescent lesions were detected intraoperatively. A total of nine fluorescent lesions, all LN clusters, were resected during surgery (Figure 1C). Mean TBR was 4.4 (SD 3.3, median 3.6, range 1.8-10.8). Apart from LNs, fluorescence was not detected elsewhere.

In addition to fluorescent lesions, biopsy samples of clinically suspect lesions and of predefined areas were analyzed on histopathology. Apart from the fluorescent primary tumor, tumor cells were not demonstrated in any of the biopsy samples, including the samples of fluorescent lesions. Therefore all fluorescent suspected metastatic lesions, all LNs, were false positives. As none of the non-fluorescent lesions contained tumor cells on histopathology either, false-negative fluorescence was not seen.

All resected LNs (n=38), including LNs that demonstrated false-positive fluorescence were assessed. FRα ex-

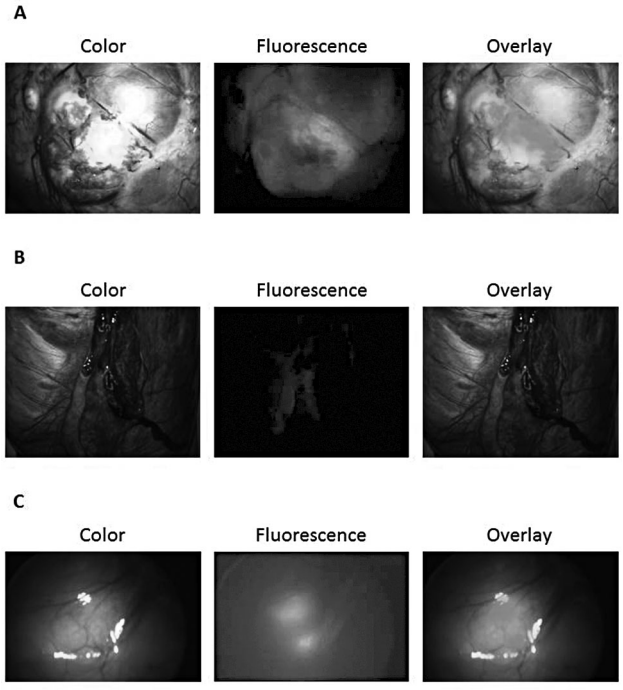

Figure 1. — Intraoperative fluorescence.
A) Clear fluorescence arising from a endometrioid adenocarcinoma. B) Absence of fluorescence in a benign mucinous cystadenoma. C) Two fluorescent lymph nodes that did not contain metastases (false-positive).

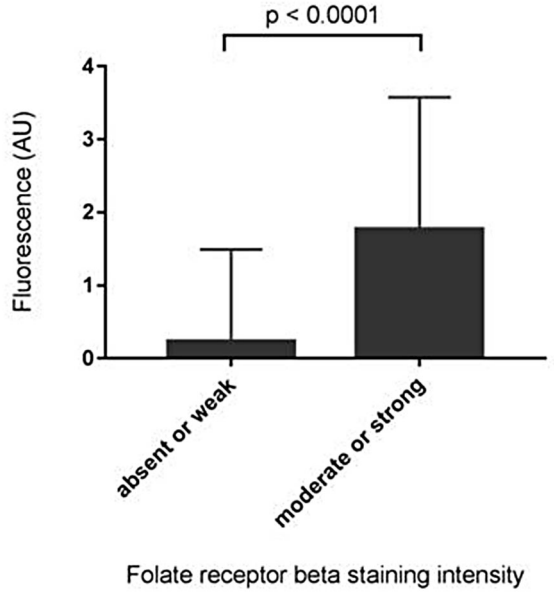

Figure 2. — Lymph node fluorescence related to folate receptor beta staining intensity. Fluorescence in lymph nodes is significantly ($p < 0.001$) related to the expression of the folate receptor beta.

pression was not seen in any of the LNs. Moderate to strong FRβ expression was seen in 29 LNs; the remaining LNs had absent or weak FRβ expression. Fluorescence signal of the LNs, measured using an imager, was significantly higher in the LNs with strong to moderate FRβ expression ($p < 0.001$) (Figure 2). The pattern of FRβ staining was concordant with the pattern of CD68 staining in all LNs with the main expression in the sinuses of the LNs (Figure 3). This typical pattern, with sparing of the follicles, was also seen on fluorescence scanning. This confirms co-localization of the fluorescence signal with the FRβ expressing macrophages (Figure 4). Additional serial sectioning and cytokeratin staining did not lead to the detection of (micro)metastases.

Discussion

NIR fluorescence imaging has the potential to detect (small) metastatic lesions that are not visible with the naked eye. This could facilitate discrimination between true early-stage and occult advanced-stage ovarian cancer. NIR fluorescence imaging could also optimize staging procedures in ovarian cancer, as targeted rather than blind biopsies can be taken, which is especially relevant in laparoscopic staging procedures, where tactile information is lacking. The use of OTL38, a NIR fluorescent FRα targeting agent, was studied in six patients that were scheduled to undergo a staging procedure for suspected early-stage ovarian cancer. Distinction between a malignant and a benign primary tumor was possible with OTL38-based intraoperative fluorescence imaging. As metastases were not present in any of these patients, the added value of OTL38 was limited in this study. In fact, apart from the primary tumor, fluorescence signal was only detected in LNs that did not contain metastases.

These false-positive LNs were detected intraoperatively in half of the patients. Because this may have implications on the applicability of OTL38 in staging procedures for ovarian cancer, the present authors studied all resected LNs in detail. In none of the LNs FRα expression was present, whereas FRβ expression was seen in the majority of the LNs. LNs with strong to moderate overexpression of FRβ had a significantly higher fluorescent signal, while fluorescent signal in LNs with weak or absent FRβ expression was very low or absent. This supports the notion that false-positive fluorescence is caused by OTL38 binding to FRβ-expressing macrophages in the LNs. Another indication for this concept is the distinct resemblance of staining pattern of FRβ with the staining pattern of CD68, a pan-macrophage marker. Alternatively, fluorescence signal arising from LNs could also be due to small metastases that were missed during routine pathology. As the presence of tumor cells is assessed on a limited number of sections stained with H&E staining, it is possible that smaller metastasis can remain undetected. To increase the likelihood of

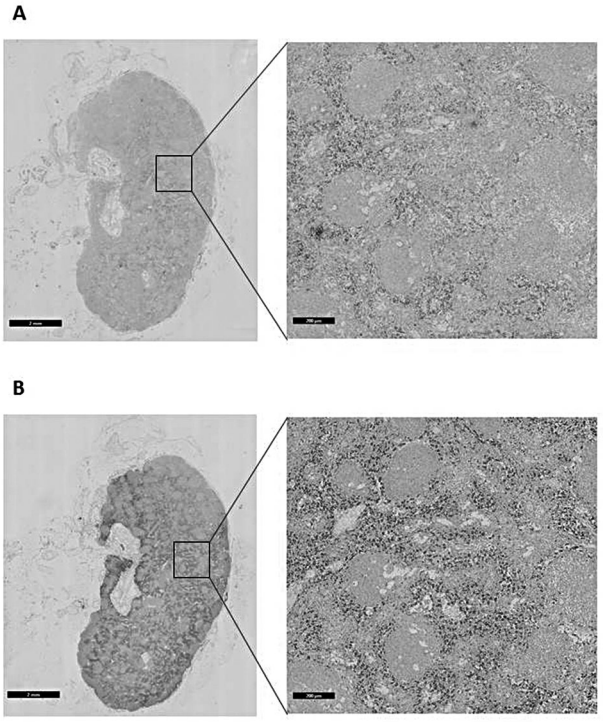

Figure 3. — Immunohistochemical staining of a false-positive lymph node. Sections of a fluorescent lymph node that does not contain metastasis stained for A) folate receptor beta and B) pan macrophage marker CD68, demonstrating a resembling staining pattern with the main expression in the, macrophage containing, sinuses of the lymph node.

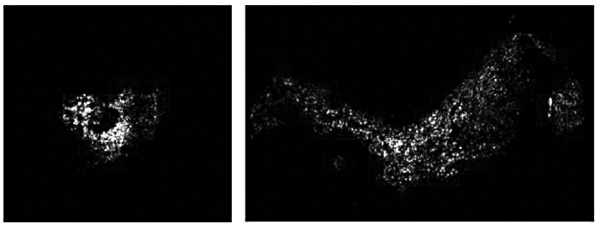

Figure 4. — Fluorescence signal of a false-positive lymph node. The images show fluorescent lymph nodes with a fluorescent pattern localized to the sinuses, consistent with FRβ staining.

detection of tumor cells, the authors performed serial sectioning and alternating H&E and cytokeratin staining of sections on all resected LNs. This detailed assessment is too time-consuming for implementation in routine practice[21]. Application of this assessment to all LNs resected during this study did not lead to the detection of additional metastases. Therefore, false-positive fluorescence in LNs is most likely a consequence of FRβ expressing macrophages in the LNs rather than missed metastases. In addition, FRβ overexpression and CD68-positive macrophages found in the LNs are likely tumor-associated macrophages (TAMs). The role of TAMs is controversial, as evidence exists for their involvement in pro- as well as anti-tumor processes. However, most recent evidence indicates that macrophages, both in the primary and metastatic sites, adopt a protumoral phenotype [22, 23]. In metastatic sides, TAMs prepare these by promoting the extravasation, survival, and persistent growth of metastatic cells. A study by Go *et al.* demonstrated that the density of TAMs in LNs was increased in micrometastasis; moreover a high density of TAMs was significantly associated with malignant LNs [24]. In light of this, resection of tumor-negative LNs that contain TAMs identified by FRβ-mediated fluorescence may even be beneficial. In fact, this may explain why resection of large numbers of negative LNs leads to survival benefits.

Accurate detection of metastatic spread in early-stage ovarian cancer is important as treatment decisions are based on the extent of disease found during surgical staging. If metastases are present but not detected during staging procedures, under-treatment of the patient will occur. Moreover, completeness of surgical staging is an independent prognostic factor for overall survival in early-stage ovarian cancer patients as completeness of staging minimizes the risk of undetected metastasis [3]. Although fluorescence imaging led to the detection of false positive lesions, metastases do not seem to be missed with NIR fluorescence imaging. High sensitivity is essential in staging procedures, as missing malignant lesions have worse implications than resection of non-malignant lesions [25]. In addition, a positive correlation between the number of resected lymph nodes during a staging procedure and overall survival is established in early-stage ovarian cancer patients and this positive effect remains when large numbers of conventionally 'negative' nodes are resected [26, 27].

To conclude, the anticipated added value of NIR fluorescence imaging using OTL38 could not be demonstrated in staging procedures for early-stage ovarian cancer, as metastatic lesions were not present in any of the patients in this small series. However fluorescent imaging using OTL38 did contribute to the resection of seemingly non-malignant LNs due to the targeting of FRβ on TAMs. However, as comprehensive lymph node sampling should be pursued in surgical staging procedures, this should not impede application of OTL38. Importantly, fluorescent imaging using OTL38 allowed distinction between a malignant and benign primary tumor and had high sensitivity, which justifies further research in a larger patient group.

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