Detection of micrometastases using SPECT/fluorescence dual-modality imaging in a CEA-expressing tumor model

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ABSTRACT

Background: Intraoperative dual-modality imaging can help the surgeon distinguish tumor from normal tissue. This technique may prove particularly valuable if small tumors need to be removed that are difficult to detect with the naked eye. The humanized anti-carcinoembryonic antigen (anti-CEA) monoclonal antibody, labetuzumab (hMN-14), can be used as a tumor targeting agent in colorectal cancer (CRC), since carcinoembryonic antigen (CEA or CEACAM5) is overexpressed in approximately 95% of CRC. Dual-labeled labetuzumab, labeled with both a near-infrared fluorescent dye (IRDye800CW) and a radioactive label (Indium-111), can be used as a tracer for dual-modality imaging. This study aimed to assess whether dual-modality imaging using Indium-111-DTPA-labetuzumab-IRDye800CW can detect pulmonary micrometastases in a mouse model. Methods: Pulmonary GW-39 human colonic carcinoma microcolonies were induced in athymic BALB/c mice by intravenous injection of 100 µL of a GW-39 cell suspension. After 1, 2, 3, and 4 weeks of tumor growth dual-modality imaging was performed 3 days after i.v. injection of Indium-111-DTPA-labetuzumab-IRDye800CW (10 µg, 25 MBq). Micro single-photon emission computed tomography (SPECT) images and optical images were acquired and image-guided surgery was performed. Finally, the biodistribution of the dual-labeled tracer was determined. Formalin-fixed sections of the lungs were analyzed using fluorescence imaging, autoradiography and immunohistochemistry. Results: Sub-millimeter pulmonary tumor colonies could be visualized with both microSPECT and fluorescence imaging from the first week of tumor growth, before they became visible with the naked eye. Furthermore, dual-modality imaging could be used to guide resection of tumor lesions. Mean uptake of the dual-labeled tracer in tumor lesions was 17.2 ± 5.4 %ID/g and 16.5 ± 4.4 %ID/g in weeks 3 and 4, respectively. Immunohistochemical analysis of the tumorous lungs showed that the distribution of the radioactive and fluorescent signal co-localized with CEA-expressing tumor lesions. Conclusion: Dual-modality imaging after injection of Indium-111-labetuzumab-IRDye800CW can be used to detect sub-millimeter CEA-expressing pulmonary tumor lesions, before they become visible with the naked eye, supporting the added value of this technique to the resection of small tumor lesions.

Key words: molecular imaging, colorectal cancer, CEA, labetuzumab, fluorescence-guided surgery.
INTRODUCTION

Radical tumor resection is crucial for optimal prognosis of cancer patients (1-6). However, during surgery small tumor lesions or positive surgical margins can be difficult to detect with the naked eye. Sensitive intraoperative imaging techniques could help the surgeon visualize these small tumor lesions that otherwise could be missed. Targeted dual-modality image-guided surgery using monoclonal antibodies tagged with both a fluorescent and a radioactive label combines the advantages of radioguided and fluorescence-guided surgery (7-14). The monoclonal antibody assures adequate targeting of a tumor-associated antigen. The radioactive label allows localization of both deeply and superficially located tumor lesions, while the fluorescent label enables the surgeon to visualize, delineate and resect the tumors with fluorescence-guided surgery.

Labetuzumab (hMN14) is a humanized monoclonal antibody that specifically recognizes carcinoembryonic antigen, which is overexpressed in approximately 95% of CRC (15). CEA is considered to be the preferred biomarker for in vivo targeting of colorectal cancer (15). In CRC incomplete tumor resection has a clearly negative effect on patients’ prognosis(16). A special surgical challenge are those patients with multiple small metastases limited to the peritoneal cavity (peritoneal carcinomatosis). In these patients long term control of disease can be achieved by cytoreductive surgery (resect all visible tumor tissue) in combination with hyperthermic intraperitoneal chemotherapy (17-21). However, the presence of tumor residue after cytoreduction negatively effects patients’ prognosis (18,20,21). Intraoperative imaging can help the surgeon limit residual disease. Rijpkema et al. have shown the feasibility of image-guided surgery with 111In-labetuzumab-IRDye800CW in mice with intraperitoneal, CEA-positive, LS174T tumors (14). However, to be of optimal benefit in the operating room, dual-modality imaging should be able to detect very small tumor lesions that can be missed with the naked eye.

The aim of the current study is to assess the feasibility of CEA-targeted dual-modality imaging to detect micrometastases. In this study, micrometastases were defined as tumor lesions smaller than 2 mm according to the 6th edition of the American Joint Committee on Cancer Cancer Staging Manual for breast cancer (22). For this aim, a model with CEA-expressing pulmonary tumor lesions was used, since it is an optimal model to study micrometastatic disease (23).
MATERIAL AND METHODS

All animal studies have been approved by the institutional Animal Welfare Committee of the Radboudumc and experiments were conducted in accordance with the principles as stated in the revised Dutch Act on Animal Experimentation (WOD 2014).

Study design

The feasibility of dual-modality imaging using Indium-111-labetuzumab-IRDye800CW to detect CEA-expressing pulmonary micrometastases was studied. Pulmonary lesions were induced in 28 athymic BALB/c mice by intravenous injection of a GW-39 cell suspension obtained by serial transplantation from subcutaneous xenografts. After 1, 2, 3, and 4 weeks of tumor growth microSPECT/CT, optical imaging and image-guided surgery were performed. Dual-modality imaging was performed 3 days after i.v. injection of Indium-111-DTPA-labetuzumab-IRDye800CW (10 µg, 25 MBq, 7 mice per timepoint). Biodistribution studies and immunohistochemical analysis of the lungs were performed.

Animal model

The experiments were performed in 28 female BALB/c nu/nu mice (6-8 weeks old, Janvier, le Genest-Saint-Isle, France). Mice were climatized to laboratory conditions for at least 1 week before use. Mice were housed in individually ventilated cages with ad libitum access to food and water. The CEA-expressing GW-39 cell line was obtained from Immunomedics, Inc. (Morris Plains, NJ). Subcutaneous GW-39 xenografts were grown to 1 cm³ and serially transplanted in BALB/c nu/nu mice. Tumors were harvested, cut into pieces, filtered through a 400-µm mesh and resuspended to a 10% cell suspension in culture medium (Roswell Park Memorial Institute medium + penicillin/streptomycin) while kept on ice, as described by Sharkey et al. The first fifteen mice were injected i.v. with 100 µL of this cell suspension with a 23-gauge needle to induce micrometastatic disease in the lungs. Three mice died during i.v. injection due to inadequate suspension of the tumor cells. Therefore, the cell suspension was filtered again through a 200-µm mesh. The remaining 13 of the total of 28 mice were injected with 100 µL of this
cell suspension without causing any health problems to the mice. Mice injected with different cell suspensions were equally distributed over the experimental groups. The cell concentration in both cell suspensions was counted by adding ZAP-OGLOBIN II lytic reagent (Beckman Coulter) to a sample, vortexing the mixture and moving the cells up and down through a 23-gauge needle. Subsequently the nuclei were counted using a Burker Turk counting chamber. The cell suspensions before and after extra filtration contained 3.7 and 3.5 million cells per mL, respectively. All animals developed pulmonary tumor lesions. Animal health was monitored on a daily basis and body weight was measured three times a week. Two weeks after injection of tumor cells, one mouse had to be taken out of the experiment prematurely because of dyspnoea and weight loss originating from excessive pulmonary tumor growth. For the same reason one animal from week 4 was scanned 2 days (instead of 3 days) after injection of the tracer. In total, 6, 7, 6, and 5 animals were used in the experiment in weeks 1, 2, 3, and 4, respectively.

**Dual-labeled antibody production**

Humanized labetuzumab (Immunoglobulin G type 1) was kindly provided by Immunomedics Europe (Darmstadt Germany). The fluorophore IRDye800CW-NHS ester was purchased from LI-COR biosciences (Lincoln, NE, USA). The bifunctional chelator ITC-DTPA was purchased from Macrocyclics (Dallas, TX, USA). First labetuzumab (10 mg/mL) was incubated in 0.1 M NaHCO₃, pH 8.5, with a 3-fold molar excess of the IRDye800CW-NHS ester (RT, 1 h). Then the mixture was incubated in 0.1 M NaHCO₃, pH 9.5, with a 20-fold molar excess of the ITC-DTPA (RT, 1 h). To remove the unconjugated IRDye800CW and ITC-DTPA, the reaction mixture was transferred into a Slide-A-Lyzer cassette (molecular weight cut-off: 20,000 Da, Thermo Scientific, Rockford, IL, USA) and extensively dialyzed against 0.25 M NH₄Ac, pH 5.5, for three days with buffer changes. The average substitution ratio (SR) of IRDye800CW molecules was 1.2/antibody as was determined by the Ultrospec 2000 UV/Visible spectrophotometer (Pharmacia Biotech, Sweden). Since GW39 cells cannot be cultured in vitro, the immunoreactive fraction of dual-labeled labetuzumab was determined after 6 h incubation with CEA-expressing LS174T cells, essentially as described by Lindmo (24), and was 90 %. DTPA-labetuzumab-IRDye800CW was stored in the dark at 4 °C until use.
Radiolabeling of DTPA-labetuzumab-IRDye800CW

DTPA-labetuzumab-IRDye800CW was incubated with 0.5 M 2-(N-morpholino)ethanesulfonic acid (MES) (30 min, RT) and $^{111}\text{InCl}_3$ (Mallinkrodt Pharmaceuticals, 's Hertogenbosch, The Netherlands). Then unincorporated Indium-111 was chelated by adding 50 mM ethylenediaminetetraacetic acid to a final concentration of 5 mM. Radiochemical purity was determined by Instant Thin Layer Chromatography on silica gel strips, using 0.1 M sodium citrate buffer, pH 6.0, as the mobile phase. Radiochemical purity always exceeded 95%. Standards of the injected dose (ID) were prepared in triplicate to be able to quantify antibody accumulation, corrected for radioactive decay for biodistribution studies.

Dual-modality imaging

Groups of mice were injected via the tail vein with Indium-111-labetuzumab-IRDye800CW (10 µg, 25 MBq $^{111}\text{In}$) (n=7 per time point). Three days later (after 1, 2, 3, or 4 weeks of tumor growth), dual-modality imaging was performed. First, mice were euthanized using O$_2$/CO$_2$ and the lungs were fixed by intratracheal injection of 4% formalin with a 21-gauge needle. Then microSPECT/CT images were obtained by scanning mice in supine position with the U-SPECT II scanner (MILabs, Utrecht, The Netherlands), using a 1.0 mm diameter multi-pinhole Ultra High Sensitive (UHS-M) collimator tube. Total scan time was 50 minutes per animal (2 time frames, 24 bed positions, scan mode: fast) for SPECT acquisition and 3 minutes for CT imaging. Subsequently, the thoraco-abdominal wall was removed surgically and mice were placed in supine position in the IVIS Lumina closed-cabinet fluorescence imager (Caliper Life Sciences, recording time 1-5 min; binning factor small or medium; F/stop 2-4; excitation filter 745 nm; emission filter ICG; Field of view C; autofluorescence [675 nm] and background correction). Fluorescence imaging was performed before and after resection of tumor lesions. Thereafter lungs were resected and scanned ex vivo in the IVIS (Field of view A) and with the Odyssey CLx flatbed fluorescence scanner (LICOR Biosciences, recording time ± 5 min; 800 nm channel; focus 1.0 mm).

Biodistribution
After dual-modality imaging the biodistribution of the radiolabel was determined. Therefore, blood, muscle, heart, spleen, kidney, pancreas, liver, stomach, duodenum, and tumors were resected and weighed. Subsequently, the amount of Indium-111 in these tissues was determined in a gamma counter (2480 WIZARD², Perkin Elmer). To correct for radioactive decay, samples were measured along with standards of the injected dose (in triplicate). The organ accumulation of Indium-111-DTPA-labetuzumab-IRDye800CW was expressed as percentage of the injected dose per gram tissue (% ID/g). Values are represented as mean uptake ± standard deviation. Statistical analyses were performed using IBM SPSS Statistics 22.0. Tissue uptake at the different timepoints was tested for significance using a one-way ANOVA test with post-hoc Bonferroni corrections. An alpha of 0.05 was used in all analyses.

**Immunohistochemical analysis**

Formalin-fixed, paraffin-embedded, 5-µm sections of the lungs were cut and analyzed autoradiographically after 1.5 weeks exposure to a phosphor imaging plate. This plate was developed using the Typhoon FLA 7000 Phosphor Imager and analyzed with Aida Image Analyzer v. 4.21. Next, fluorescence imaging of the pulmonary sections was performed with the Odyssey CLx flatbed fluorescence scanner (800 nm channel, recording time 1-5 min, focus 1.0 mm). Subsequently, sections were stained for CEA with humanized labetuzumab, as described previously by Schoffelen et al. and counterstained with Hematoxylin (25). Furthermore, on adjacent tissue sections a standard Hematoxylin & Eosin staining was performed.
RESULTS

Dual-modality imaging

Sub-millimeter pulmonary tumor lesions could be visualized with dual-modality imaging in all animals that were imaged from the first week of tumor growth onwards (Fig. 1; Supplemental Fig 1). MicroSPECT/CT showed uptake of dual-labeled labetuzumab in pulmonary tumor lesions and limited uptake in the liver, spleen and lymph nodes (Figs. 1A and 1B). After resection of the thoraco-abdominal wall, no tumor lesions were visible with the naked eye after one week of tumor growth (Fig. 1C). However, superficially-located tumor lesions smaller than 1 mm were identified with fluorescence imaging (Figs. 1D and 1E). The presence of these CEA-expressing tumor lesions was confirmed by immunohistochemistry and the distribution of the radioactive and fluorescent signal in tissue sections (autoradiography and flatbed fluorescence imaging) co-localized with CEA-expressing tumor lesions (Fig. 2; Supplemental Figs. 2 and 3). Pulmonary tumor lesions became visible by macroscopic inspection after two weeks of tumor growth. However, CEA-targeted dual-modality imaging revealed additional pulmonary tumor lesions that had been missed by macroscopic inspection (Supplemental Fig. 4). No metastases outside the lungs were seen. After 3 or 4 weeks of tumor growth, tumor lesions were clearly visible by the naked eye and were visualized by dual-modality imaging.

Fluorescence-guided surgery

Superficially-located pulmonary lesions identified with fluorescence imaging were subsequently resected based on their fluorescent signal (Fig. 3; Supplemental Fig. 5). In this example (three weeks after tumor induction), the lungs were imaged again after resection of the tumor lesions to confirm that a radical tumor resection had been performed (compare Figs. 3C and 3D). Autoradiography of the tissue sections and immunohistochemistry did not identify remaining tumor lesions (Fig. 3E).

Biodistribution

One and 2 weeks after tumor induction, quantification of tumor uptake was unreliable because the tumor weight could not be determined accurately. In weeks 3 and 4, mean uptake of the dual-labeled tracer in
tumor lesions was 17.2 ± 5.4 %ID/g and 16.5 ± 4.4 %ID/g, respectively. Blood levels of the dual-labeled tracer decreased from 16.5 ± 2.8 %ID/g in the group that was imaged 1 week after tumor induction to 7.7 ± 1.3 %ID/g in the group that was imaged 4 weeks after tumor induction. Tracer uptake in the other organs is shown in figure 4 and supplemental Table 1 and expressed as mean ± standard deviation. Apart from the liver, uptake in all organs was significantly lower at week 4 compared to week 1.
DISCUSSION

This study shows the potential of CEA-targeted dual-modality imaging using dual-labeled labetuzumab to detect CEA-expressing micrometastases before they become visible with the naked eye. Furthermore, it shows that dual-labeled labetuzumab can be used to perform image-guided surgery of very small tumor lesions. These results support the added value of dual-modality imaging to visualize and resect small tumor lesions.

Labetuzumab is a humanized monoclonal antibody that specifically recognizes CEACAM5. Clinical studies have shown the potential of radiolabeled anti-CEA antibodies for SPECT imaging and radioimmunotherapy, but image-guided surgery with dual-labeled labetuzumab still awaits clinical translation (26,27). In a previous study, we showed the feasibility of image-guided surgery with 111In-labetuzumab-IRDye800CW in mice with intraperitoneal CEA-positive LS174T tumors (14). In the current study, we show the added value of dual-modality imaging by assessing the feasibility of detecting micrometastases. For this purpose, the GW-39 model was chosen, since it is a well established CRC model for studying CEA-expressing micrometastatic disease in the lungs (23). The finding that dual-modality imaging can detect micrometastatic disease is very promising. Although the role for surgery in pulmonary metastases of colorectal cancer origin is limited, the current results could also apply to other CEA-expressing cancers or, when using a different tumor-targeting agent, to other types of cancer; e.g., dual-labeled farletuzumab (anti-folate receptor alpha) in ovarian cancer surgery. The main role for surgery in metastasized CRC patients is in patients with metastases that are limited to the peritoneal cavity, and in a subgroup of patients with limited liver metastases. Patients with peritoneal carcinomatosis represent a specific surgical challenge, since lesions can be very small and numerous and difficult to differentiate from scar tissue from previous surgery. In this study we have demonstrated that CEA-expressing micrometastases can be detected sensitively with the dual-modality imaging agent. In peritoneal carcinomatosis, tumor lesions are located superficially on the abdominal organs instead of distributed within a solid organ, such as in our pulmonary model. Together with the mobility of the intra-abdominal organs (e.g., intestines), this may simplify the use of dual-modality imaging in the abdominal setting compared to the pulmonary setting. The high liver uptake of Indium-111-labetuzumab-IRDye800CW and the prolonged retention of Indium-111 in the liver may limit the role of the dual-labeled agent in the
detection of micrometastases in or near the liver. Clinical trials with Indium-111-labeled antibodies in other tumor types have shown that imaging of liver metastases with Indium-111-labeled monoclonal antibodies is feasible (28,29). However, the addition of a fluorescent label (IRDye800CW) may reduce the tumor-to-liver contrast, due to the enhanced uptake in the liver (30). Therefore, dual-modality imaging using Indium-111-labetuzumab-IRDye800CW may not be useful for intraoperative detection of small liver metastases.

In the current study sub-millimeter pulmonary tumor colonies could be visualized with dual-modality imaging before they became visible with the naked eye. After 3 and 4 weeks of tumor growth, image-guided resection of tumor lesions was performed. Figure 3 demonstrates that dual-modality imaging can be used to check if a complete tumor resection has been performed. Since most of the mice had numerous pulmonary tumor nodules, resection of all tumor lesions was challenging in this tumor model. Biodistribution studies showed that the mean uptake of dual-labeled labetuzumab in tumor lesions was 17.2 ± 5.4 %ID/g and 16.5 ± 4.4 %ID/g in weeks 3 and 4, respectively. Mean blood levels of dual-labeled labetuzumab decreased gradually from week 1 to week 4 after tumor induction (16.5, 12.1, 9.3 and 7.7 %ID/g in week 1, 2, 3, and 4, respectively). An explanation might be that there is increased shedding of CEA into the blood by tumors in mice with a higher tumor load and therefore faster clearance of the dual-labeled antibody as was described previously (31,32). Sharkey et al. reported that in the presence of high plasma CEA levels (> 400 ng/ml), there is significant complexation of the antibody and that patients with elevated CEA levels have faster blood clearance of radiolabeled labetuzumab (33). Complexation could be reduced by increasing the protein dose (33). This phenomenon has to be considered when applying CEA-targeted imaging with dual-labeled labetuzumab in patients. An alternative is to exclude patients with very high plasma CEA levels.

The advantage of a dual-labeled tracer is that the same tracer can be used for preoperative and intraoperative imaging. Preoperative CT and/or Fluorine-18 fluorodeoxyglucose positron emission tomography fail to detect peritoneal carcinomatosis in approximately 9% and 17% of patients, respectively (34). The Indium-111-labetuzumab-IRDye800CW SPECT/CT might be used to detect CRC metastases preoperatively. Intraoperatively tumors can be localized with a gamma probe and subsequently a fluorescence camera can be used to guide and facilitate tumor resection and to assess the surgical cavity for remnant disease. That both techniques are complementary to each other is illustrated in figures 1 and
3. Due to the limited penetration depth of light, only superficially located tumor lesions can be detected with fluorescence imaging (Figs. 1D,E and 3B,D). However, the preoperative SPECT/CT reveals that tumor load is more extensive and in the human situation a gamma probe can be used together with a fluorescence camera for intraoperative tumor detection. The dual-labeled approach ensures that both signals originate from the same molecule in contrast to co-injection of a radiolabeled and a fluorescently-labeled agent.

Although this study demonstrates the feasibility of dual-modality imaging of very small CEA-expressing tumors, there are some limitations that have to be considered. Since imaging was performed in euthanized animals, direct translation of these results to the human situation is premature. Also, one should consider that the sensitivity of real-time fluorescence imaging systems used in the clinic differs significantly from the systems that were used in this study. Several reports have shown the feasibility of targeted fluorescence imaging of cancer in humans (8,35,36); however, the technique is still in its infancy and has to be optimized before it can be used in clinical practice. Another consideration is that the spatial resolution of images acquired with clinical PET scanners is higher than the resolution of SPECT scanners. Therefore, for preoperative imaging purposes, the use of a positron-emitting radionuclide, e.g., Zirconium-89, could be beneficial. However, the 511 keV gamma rays are less suitable for intraoperative detection with a standard gamma probe. Therefore, the optimal tracer has to be selected depending on the clinical question.

**CONCLUSION**

In summary, this study shows the potential of CEA-targeted dual-modality imaging using dual-labeled labetuzumab to detect and resect CEA-expressing micrometastases. These data support the initiation of a feasibility study of dual-modality, image-guided surgery with Indium-111-DTPA-labetuzumab-IRDye800CW in patients with peritoneal carcinomatosis of colorectal origin.
FINANCIAL DISCLOSURES

Dr. D.M. Goldenberg is an officer of Immunomedics, and is an inventor on patents related to this technology.
REFERENCES


FIGURES

Figure 1: Example of dual-modality imaging in a mouse one week after tumor cell injection. Pulmonary tumor lesions could be visualized with microSPECT/CT (coronal view A, sagittal view B). Some physiologic uptake of the tracer in the liver and lymph nodes is observed. After resection of the thoraco-abdominal wall, no tumor lesions were visible with the naked eye (C). Fluorescence imaging with the IVIS lumina closed cabinet fluorescence camera system could identify the superficially located tumor lesions (D). Fluorescence imaging with the Odyssey flatbed fluorescence scanner of the resected lungs could visualize numerous pulmonary lesions (E). L = lung, H = heart.
Figure 2: Autoradiography (A), fluorescence imaging (C), and immunohistochemistry; CEA (B) and H&E (D) of 5-µm paraffin-embedded pulmonary sections confirmed the presence of sub-millimeter tumor lesions in the lungs after one week of tumor growth. The fluorescent (C) and radioactive signals (A) co-localize with CEA-expressing tumor lesions (B).
Figure 3: Image-guided surgery using dual-labeled labetuzumab in week 3. In this example microSPECT/CT (A) did not reveal any pulmonary lesion, probably due to their location close to the liver. Fluorescence imaging revealed one superficial pulmonary nodule (D), while fluorescence imaging of the resected lungs revealed a second nodule behind the lung (B). Both lesions were resected using fluorescence-guided surgery (C), and CEA staining of lung sections did not reveal remaining lesions (E).
Figure 4: Biodistribution of Indium-111-DTPA-labetuzumab-IRDye800CW at 72 hours after injection at 1, 2, 3, and 4 weeks after tumor induction. One and 2 weeks after tumor induction, tumors were too small for reliable quantification, since the tumor weight could not be determined accurately. Apart from the liver, uptake in all organs was significantly lower in week 4 compared to week 1. Tissue uptake is expressed as % ID/g. Values represent mean ± SD.
Supplemental Figure 1: Overview of dual-modality imaging (upper row microSPECT/CT, lower row Odyssey fluorescence imaging of resected lungs) in mice after 2 (A,D), 3 (B,E) and 4 (C,F) weeks of tumor growth.
Supplemental Figure 2: Representative examples of sets (fluorescence imaging, autoradiography and CEA-staining) of adjacent slices of pulmonary metastases from mice from week 2 (A-C), week 3 (D-F) and week 4 (G-I). Fluorescence imaging with a flatbed fluorescence scanner and autoradiography of 5-µm sections of lung tissue show the co-localization of dual-labeled labetuzumab in CEA-expressing tumor lesions. During the experiment, the tumors showed increasing central necrosis and therefore uptake of dual-labeled labetuzumab was mainly in the periphery of the tumor lesions (see supplemental Figure 3).
Supplemental Figure 3: Autoradiography shows mainly uptake of dual-labeled labetuzumab in the periphery of the tumor lesion (A). Extensive central necrosis is seen after 3 weeks of tumor growth on the corresponding H&E-staining (B).
Supplemental Figure 4: *Ex vivo* example of the resected lungs and heart after 2 weeks of tumor growth. Pulmonary tumor lesions are now visible with the naked eye (white circles); however, CEA-targeted fluorescence imaging reveals multiple additional superficial pulmonary tumor lesions (histologically confirmed).
Supplemental Figure 5: Image-guided surgery after 4 weeks of tumor growth: microSPECT/CT (A), fluorescence imaging (B), fluorescence imaging after partial resection of tumor nodules (C). Due to the high tumor density, a complete resection of all tumor nodules was not possible in this case.
Supplemental Table 1:

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<td>16.5 (4.4)</td>
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Values are expressed as mean (+SD) % ID/g. Tumor uptake could not be reliably quantified after 1 and 2 weeks of tumor growth.
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