Radionuclide and Fluorescence Imaging of Clear Cell Renal Cell Carcinoma Using Dual Labeled Anti-Carbonic Anhydrase IX Antibody G250

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Abbreviations and Acronyms

CAIX = carbonic anhydrase IX

ccRCC = clear cell renal cell carcinoma

CT = computerized tomography

DTPA = diethylenetriamine-pentaacetic acid

ID = injected dose

NIR = near infrared

SPECT = single photon emission CT

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Study received Radboud University Nijmegen institutional animal welfare committee approval.

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Purpose: Tumor targeted optical imaging using antibodies labeled with near infrared fluorophores is a sensitive imaging modality that might be used during surgery to assure complete removal of malignant tissue. We evaluated the feasibility of dual modality imaging and image guided surgery with the dual labeled anti-carbonic anhydrase IX antibody preparation ^{111}In-DTPA-G250-IRDye800CW in mice with intraperitoneal clear cell renal cell carcinoma.

Materials and Methods: BALB/c nu/nu mice with intraperitoneal SK-RC-52 lesions received 10 μg DTPA-G250-IRDye800CW labeled with 15 MBq ^{111}In or 10 μg of the dual labeled irrelevant control antibody NUH-82 (20 mice each). To evaluate when tumors could be detected, 4 mice per group were imaged weekly during 5 weeks with single photon emission computerized tomography/computerized tomography and the fluorescence imaging followed by ex vivo biodistribution studies.

Results: As early as 1 week after tumor cell inoculation single photon emission computerized tomography and fluorescence images showed clear delineation of intraperitoneal clear cell renal cell carcinoma with good concordance between single photon emission computerized tomography/computerized tomography and fluorescence images. The high and specific accumulation of the dual labeled antibody conjugate in tumors was confirmed in the biodistribution studies. Maximum tumor uptake was observed 1 week after inoculation (mean ± SD 58.5% ± 18.7% vs 5.6% ± 2.3% injected dose per gm for DTPA-G250-IRDye800CW vs NUH-82, respectively). High tumor uptake was also observed at other time points.

Conclusions: This study demonstrates the feasibility of dual modality imaging with dual labeled antibody ^{111}In-DTPA-G250-IRDye800CW in a clear cell renal cell carcinoma model. Results indicate that preoperative and intraoperative detection of carbonic anhydrase IX expressing tumors, positive resection margins and metastasis might be feasible with this approach.

Key Words: kidney; carcinoma, renal cell; G250 monoclonal antibody; tomography, emission-computed, single-photon; surgical procedures, operative

To radically excise tumor tissue in oncologic surgery intraoperative imaging may be applied. Tumor targeting molecules labeled with NIR dyes can aid in the intraoperative detection and accurate delineation of malignant

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lesions, thus, enabling image guided surgery of primary tumors and metastasis, and the detection of positive resection margins.[1] A major drawback of fluorescence imaging techniques is the limited penetration depth of emitted light in biological tissue. By combining radionuclide and fluorescent tracers a powerful complementary imaging system that overcomes the limitations of each individual modality can be developed for improved in vivo detection of tumors.[2–4]

The fluorophore IRDye800CW is a NIR dye manufactured under CGMP (Current Good Manufacturing Practices) that is approved for use in clinical trials. This fluorescent dye emits NIR 789 nm photons when excited at 774 nm. It can be coupled to tumor specific targeting molecules such as mAbs and detected intraoperatively with a NIR imaging camera.

For ccRCC, which accounts for approximately 85% of malignant renal tumors, the chimeric mAb G250 (also known as girentuximab) is the most promising targeting mAb known. The use of G250 in radioimmunodetection and radioimmunotherapy to detect or treat ccRCC has been investigated extensively in the preclinical and clinical settings.[5,6] G250 specifically recognizes CAIX, a tumor associated antigen ubiquitously expressed in primary ccRCC and its metastases. Because of the high and specific targeting of G250 for all CAIX positive lesions (primary tumors and metastasis), this antibody could act as a potent carrier to deliver radiotracer and NIR dye to these lesions. As we recently described, such a dual modality imaging system enables preoperative SPECT and intraoperative tumor detection by the radioactive and NIR signals.[7]

We describe ^{111}In-DTPA-G250-IRDye800CW and report the feasibility of dual modality imaging and image guided surgery in an intraperitoneal ccRCC model. This might ultimately enable in vivo detection of primary ccRCC, positive resection margins or distant metastasis in the future.

MATERIALS AND METHODS

ccRCC in Nude Mice

Animals. All experiments were performed in accordance with the principles of the Dutch Act on Animal Experimentation, revised 1997, and approved by the Radboud University Nijmegen institutional animal welfare committee. Animals were housed and fed according to Dutch animal welfare regulations. Experiments were performed in 8 to 10-week-old female nude BALB/c nu/nu mice (Janvier, le Genest-Saint-Isle, France) weighing 20 to 25 gm. Mice were accustomed to laboratory conditions for at least 1 week before experimental use. They were housed under nonsterile standard conditions in filter topped cages with free access to chlorophyll-free animal nutrition (ssniff, Soest, Germany) and water.

Cell Lines and Tumor Induction. Intraperitoneal tumor growth was induced by intraperitoneal injection of 0.2 ml of a suspension of 3×10^6 SK-RC-52 cells (Memorial Sloan Kettering Cancer Center, New York, New York). This resulted in tumor nodules in the peritoneal cavity ranging from a submillimeter size to 5 mm in diameter after 1 to 5 weeks in accord with our previous experience with this model.[8] SK-RC-52 is a CAIX expressing human ccRCC cell line previously described by Ebert et al.[9] In August 2013 the cell line was tested for authenticity by short tandem repeat profiling. As we recently described in our results with dual labeled girentuximab in multiple tumor cell lines,[7] no additional studies with a CAIX negative control tumor group were performed in this study.

Study Design. Athymic female BALB/c nu/nu mice were injected intraperitoneally with 3×10^6 SK-RC-52 cells as previously described.[8] After tumor cell inoculation the mice were divided into 2 groups of 20 each. Based on our previous results in this intraperitoneal xenograft model[8] we used an antibody protein dose of 10 μg. One group was injected intravenously with 10 μg DTPA-G250-IRDye800CW labeled with 15 MBq ^{111}In before imaging and the other group received 10 μg of the dual labeled, irrelevant isotype matched control antibody NUH-82.[10] To evaluate when tumors could be detected 4 mice per group were imaged with microSPECT/CT and with the IVIS® Lumina optical imager 48 hours after injection weekly.

Imaging Agents and Labeling

Antibodies. G250 is a murine IgG1 mAb directed against the CAIX antigen expressed in ccRCC. NUH-82, a murine IgG1 mAb not directed against any known antigen in nude mice, served as a negative control.

Conjugation, Radiolabeling and Fluorescent Labeling. G250 and NUH-82 were conjugated with IRDye800CW-NHS (LI-COR®) according to the supplier protocol. Briefly, 1 mg mAb was incubated with a sixfold excess of IRDye800CW and stirred for 3 hours at room temperature. After solving the mixture in a 0.1 M sodium carbonate buffer (pH 8.5) it was purified using a PD-10 column.

After conjugation with IRDye800CW mAb G250 and NUH-82 were conjugated with DTPA as described previously.[11] The reaction mixture was transferred to a Slide-A-Lyzer™ cassette (molecular weight cutoff 20,000 Da) and extensively dialyzed against 0.25 M ammonium acetate (pH 5.4) for 3 days to remove unconjugated IRDye800CW and DTPA. An average of 1.1 IRDye800CW molecules was conjugated per mAb molecule as determined by spectrophotometry. The dual labeled antibody preparations were stored in the dark at 4C until use.

G250 (10 μg) was dissolved in 0.25 M ammonium acetate buffer (pH 5.4) and radiolabeled with 15 MBq $^{111}InCl_3$ in 2 volumes of 0.1 M MES (2-[N-Morpholino] ethanesulfonic acid hemisodium salt) (Sigma-Aldrich®). After 20-minute incubation at room temperature 50 mM ethylenediaminetetraacetic acid (Sigma-Aldrich) were added to a final concentration of 5 mM. Radiochemical purity was determined by instant thin layer

chromatography on silica gel using 20 mM sodium citrate buffer (pH 5.0) as the mobile phase.

SPECT/CT and Fluorescence Imaging. Imaging with the U-SPECT II scanner (MILabs, Utrecht, The Netherlands) was performed 48 hours after injection of the dual labeled antibody preparations via the tail vein. A 1.0 mm diameter multi-pinhole UHS-M collimator tube (MILabs) was used. Mice were anesthetized with a mixture of oxygen, N_2O and isoflurane, and placed supine in the scanner. Body temperature was maintained at 37C during the scan. Total scan time was approximately 60 minutes per mouse for SPECT acquisition and 3 minutes for CT. Images were reconstructed using software (MILabs).

Immediately after completing image acquisition the mice were sacrificed and placed supine in the optical scanner after removing the abdominal skin. Optical images were acquired with the IVIS Lumina imaging system (recording time 1 to 3 minutes, binning factor medium, emission filter ICG, field of view C and excitation filter 745 nm).

Tumor Specimen Processing. After dissection the tumor specimens were processed and 10 μm sections were cut. Ex vivo autoradiogram studies were performed by exposing sections to a BAS-SR 2025 imaging plate (Fujifilm, Valhalla, New York) for 1 hour. Images were acquired with a BAS 1800 II system radioluminogram laser imager (Fujifilm) and analyzed with Aida Image Analyzer software (Raytest, Straubenhardt, Germany). Sections were subsequently measured with the Odyssey 9120 fluorescence imaging system (LI-COR) (800 nm channel and 42 μm resolution). CAIX expression was detected by staining with anti-CAIX mouse mAb M75, a hybridoma supernatant obtained from the HB-11128 cell line (ATCC®).

Biodistribution Studies. After SPECT and optical image acquisition the mice were sacrificed and tissues were dissected for ex vivo biodistribution studies. A tumor sample and samples of normal tissues (blood, muscle, heart, lung, spleen, pancreas, stomach, duodenum, kidney and liver) were dissected, weighed and counted in a 1480 Wizard® 3″ γ counter. Injection standards were also counted to correct for radioactive decay. Tissue uptake of the dual labeled antibody is shown as the percent of ID/gm.

Statistical Methods. Statistical analysis was performed with SPSS® Statistics, version 20.0 and Prism®, version 5.03. Differences in the uptake of dual labeled antibodies determined from biodistribution studies were tested for significance using the t-test with differences considered significant at 2-sided $p < 0.05$. All values are shown as the mean ± SD.

RESULTS

SPECT/CT and optical images acquired 48 hours after injecting ^{111}In-DTPA-G250-IRDye800CW clearly delineated the intraperitoneal ccRCC expressing CAIX. As early as 1 week after tumor cell inoculation the lesions were clearly visualized with each imaging modality in 3 of the 4 mice (fig. 1). In the mouse in which SPECT/CT and optical imaging did not indicate tumor no macroscopic tumor was found during meticulous examination of the peritoneal cavity. Although deeper lying lesions (eg subhepatic or mesenteric) could not be detected by optical imaging due to the limited penetration depth of the emitted light, there was good concordance between SPECT/CT and optical images of more superficial tumors (fig. 1).

All abdominal tumors detected by optical imaging were subsequently removed. After removal no residual tumor was detected by optical imaging or

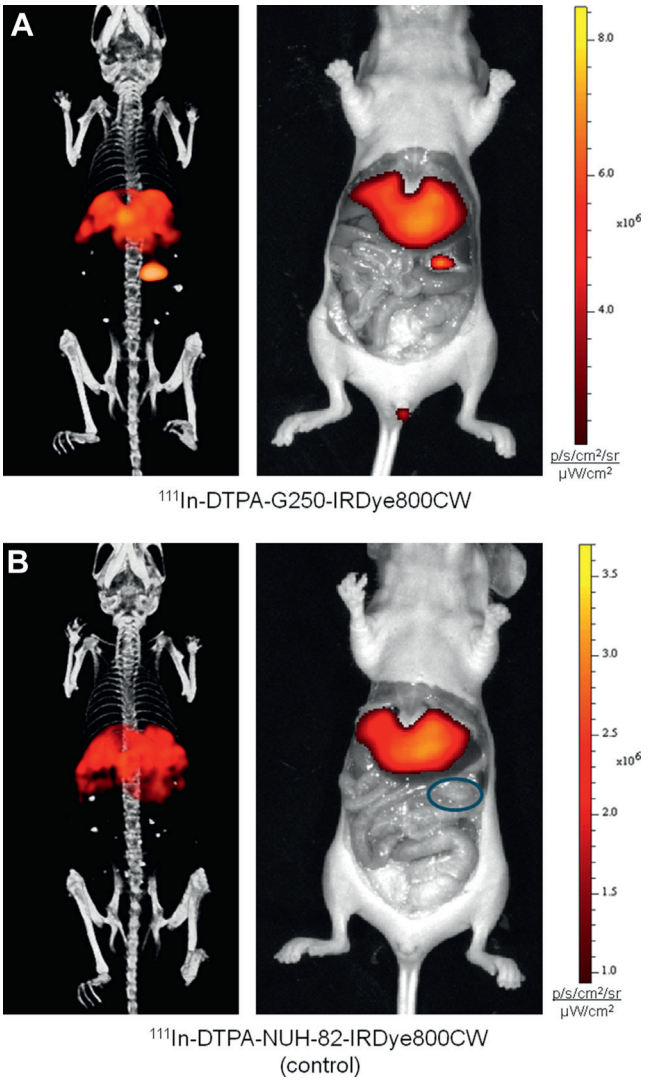

Figure 1. SPECT/CT and fluorescence images of mice 1 week after tumor cell inoculation clearly reveal intraperitoneal ccRCC 48 hours after injection of dual labeled imaging probe ^{111}In-DTPA-G250-IRDye800CW (*A*). Note some physiological uptake of tracer in liver and retention of catabolic products in bladder and urine. In mouse that received dual labeled NUH-82 parasplenic lesion was detected macroscopically (blue oval) but not by either imaging modality (*B*).

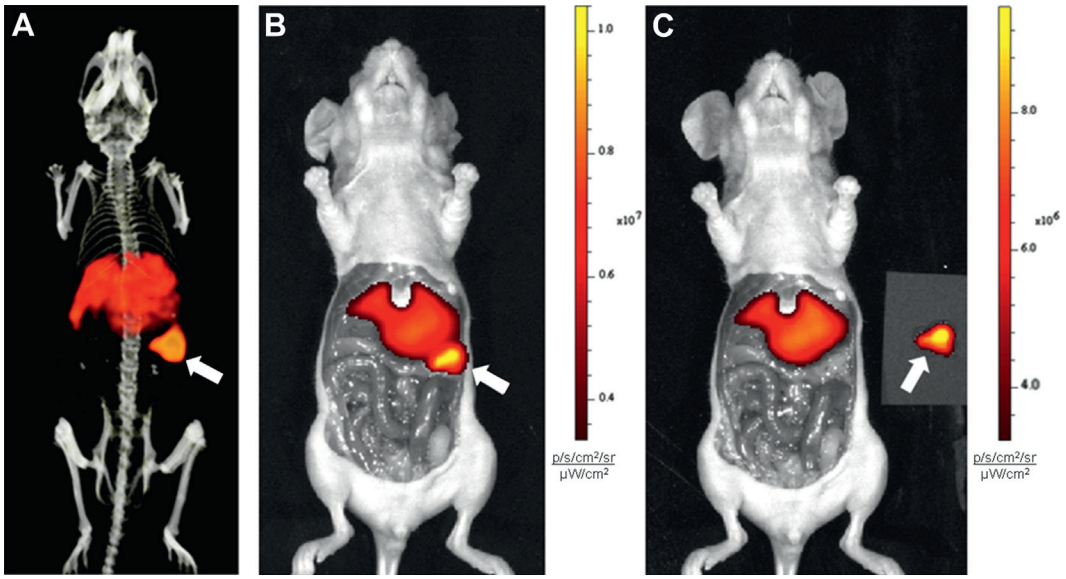

Figure 2. Preoperative SPECT/CT (*A*) and fluorescence (*B*) images of mouse with intraperitoneal ccRCC 48 hours after injection of tumor targeting, dual labeled imaging probe ^{111}In-DTPA-G250-IRDye800CW. Note some physiological tracer uptake in liver. Abdominal tumor (arrow) was subsequently removed by fluorescence image guided surgery (*C*). After resection no residual tumor was detected macroscopically or by optical imaging.

macroscopically (fig. 2). Some physiological uptake of conjugate in the liver was observed and retention of catabolism products was detected in the bladder. Excellent concordance was noted between autoradiogram and fluorescence imaging of tumor sections (fig. 3).

High and specific accumulation of ^{111}In-DTPA-G250-IRDye800CW in CAIX expressing tumors was confirmed by biodistribution data (fig. 4). The highest tumor uptake of ^{111}In-DTPA-G250-IRDye800CW (58.5% ± 18.7% ID/gm and mean tumor weight 0.02 ± 0.01 gm) was observed 1 week after tumor cell inoculation. One week after inoculation mice injected with the isotype matched, irrelevant ^{111}In-DTPA-NUH-82-IRDye800CW conjugate demonstrated significantly lower tumor uptake (5.6% ± 2.3% ID/gm, p = 0.008). Mean tumor uptake of ^{111}In-DTPA-G250-IRDye800CW was 49.2% ± 15.7% ID/gm (mean tumor weight 0.03 ± 0.01 gm), 47.9% ± 5.5% ID/gm (0.06 ± 0.02 gm), 38.6% ± 16.3% ID/gm (0.05 ± 0.01 gm) and 17.6% ± 7.7% ID/gm (0.14 ± 0.08 gm) at 2, 3, 4 and 5 weeks, respectively. In addition, blood levels decreased and liver uptake increased during the course of the experiment (fig. 4).

The tumor-to-liver ratio steadily decreased from 2.55 after 1 week to 0.36 5 weeks after tumor induction, resulting in a tumor-to-liver ratio of 2.16, 1.95 and 1.13 at 2, 3 and 4 weeks, respectively. These values were significantly higher than at 5 weeks after inoculation (p = 0.005, 0.013 and 0.004, respectively).

DISCUSSION

In this study dual modality imaging and image guided surgery of tumors with the dual labeled tracer ^{111}In-DTPA-G250-IRDye800CW was tested

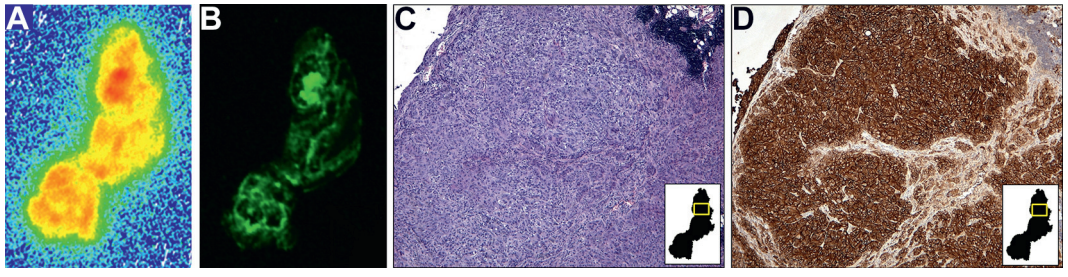

Figure 3. Autoradiogram (*A*) and fluorescence (*B*) images of tumor section 48 hours after ^{111}In-DTPA-G250-IRDye800CW injection. Note enhanced tracer uptake in vital tumor region (*C*). H&E, reduced from ×40. Also note high CAIX expression (*D*). M75 staining, reduced from ×40.

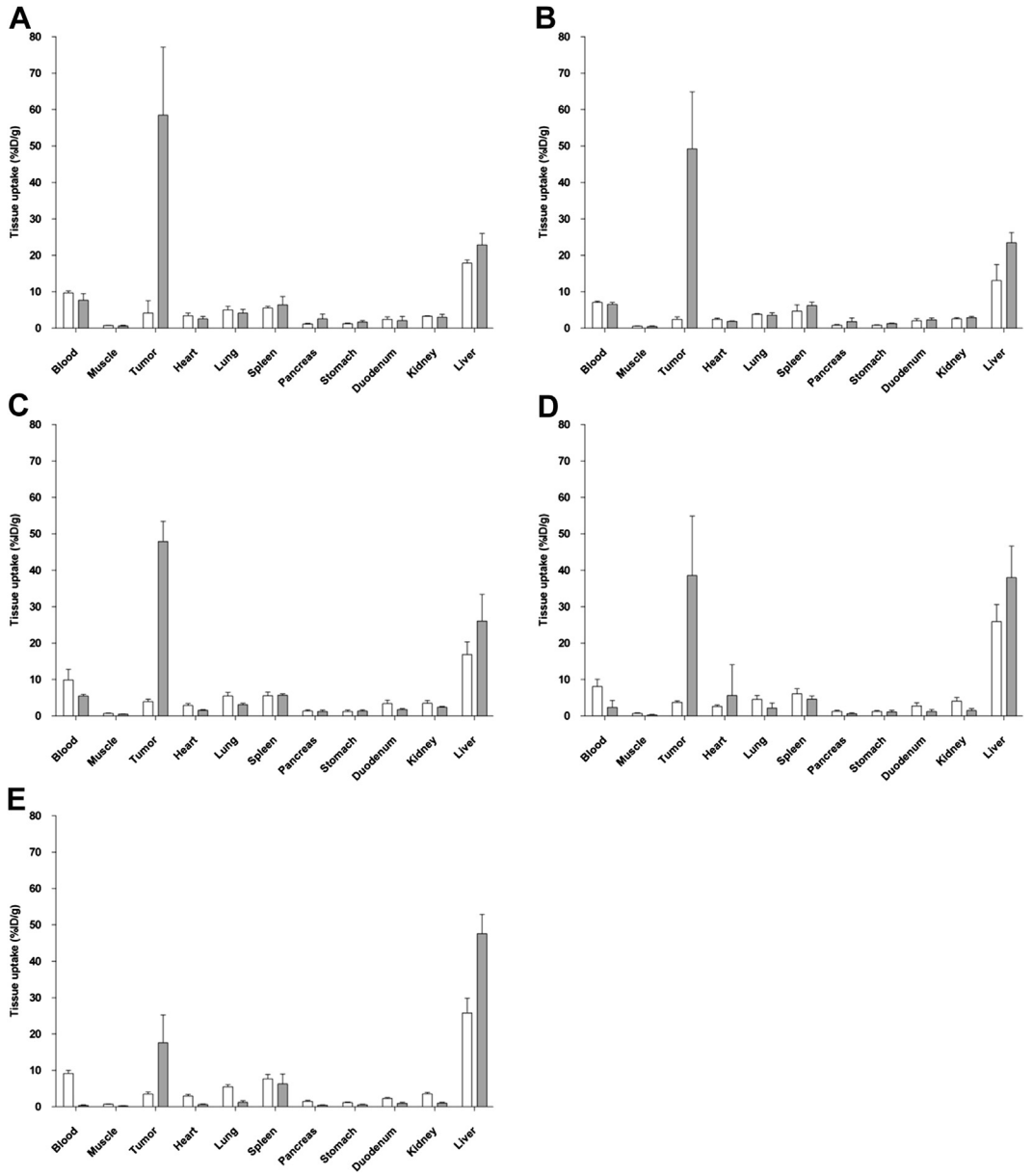

Figure 4. Mean ± SD biodistribution of dual labeled antibody ^{111}In-DTPA-G250-IRDye800CW (gray bars) and ^{111}In-DTPA-NUH-82-IRDye800CW (open bars) 48 hours following injection in mice 1 (*A*), 2 (*B*), 3 (*C*), 4 (*D*) and 5 (*E*) weeks after tumor induction shows specific tumor targeting. Note decreasing tumor and blood levels, and increasing liver uptake with time.

in a ccRCC model. SPECT and fluorescence images revealed high and specific accumulation of dual labeled G250 in CAIX expressing lesions with good concordance between the 2 modalities (fig. 1). After image guided surgery no residual tumor was detected macroscopically or by optical imaging (fig. 2). This indicates that preoperative and intraoperative detection of CAIX expressing lesions might be feasible with this tracer.

The high and specific accumulation of ^{111}In-DTPA-G250-IRDye800CW in CAIX expressing tumors was confirmed by biodistribution studies (fig. 4). Tracer accumulation in the lesions depended highly on the total tumor load in accord with previous studies.[8,12] Especially in mice with a high tumor load at 5 weeks after tumor cell inoculation tumor uptake was significantly less than tumor uptake in mice with a lower tumor burden. In the course of the experiment we observed a gradually decreasing tumor accumulation along with lower blood levels and increasing liver uptake (fig. 4). In addition, the tumor-to-liver ratio decreased dramatically (fig. 4). This could have resulted from antibody conjugate binding to circulating antigen in the blood,[13] which led to rapid clearance of the antibody-antigen immune complexes via the liver.

Our results indicate that intraoperative detection of primary ccRCC and positive resection margins might be feasible in patients. Positive resection margins could theoretically be detected intracorporeally or by ex vivo examination of the surgical specimen during surgery. Moreover, G250 based fluorescence imaging can potentially be used for metastasectomy in patients with a history of ccRCC.

An important advantage of the currently used mAb G250 is that it has been studied extensively in clinical trials[14–20] and adjuvant setting for nonmetastatic disease (ClinicalTrials.gov NCT00087022). This indicates that clinical translation of this dual labeled tracer is feasible. In addition, labeling the antibody preparation with ^{111}In enables high quality, preoperative whole body SPECT, which can help with clinical decision making in these patients.[18]

Although the results of the current study are promising, several parameters may affect ccRCC visualization. 1) Targeting the antibody and, thus, the intensity of the radioactive and the fluorescent signal depends on the degree and extent of CAIX expression. Although CAIX expression is homogeneous in most ccRCCs, G250 uptake is quite heterogeneous due to differences in perfusion, vascular permeability and interstitial fluid pressure.[19,21] As a result, uptake of the G250 based tracer may vary substantially among lesions.

2) The antibody-to-dye molar substitution ratio strongly affects the in vivo characteristics of the antibody conjugate, including biological properties, signal-to-background ratio, clearance and biodistribution.[22,23] Indeed, at higher substitution ratios (3 or greater) rapid blood clearance and high liver uptake were observed (data not shown).

3) The IVIS Lumina setup differs from currently available clinical surgical imaging systems. Although intraperitoneal ccRCC was clearly visualized, we do not know whether the sensitivity of the preclinical IVIS Lumina system is comparable to that of the clinical systems. Therefore, it is unclear whether real-time imaging can be performed in the operating room.

In the near future a phase I clinical trial with ^{111}In-girentuximab-IRDye800CW is planned. The main objective of this trial will be to assess the safety and feasibility of dual modality imaging using this dual labeled antibody in patients with a primary renal tumor suspicious for ccRCC. In case ccRCC is detected by the 2 modalities in the clinical setting, additional studies are warranted to evaluate the validity and cost-effectiveness of this approach.

CONCLUSIONS

We evaluated the feasibility of dual modality imaging with the dual labeled antibody preparation ^{111}In-DTPA-G250-IRDye800CW in a model of intraperitoneal ccRCC xenografts. SPECT and fluorescence images clearly revealed specific accumulation of ^{111}In-DTPA-G250-IRDye800CW in ccRCC with good concordance between the 2 imaging modalities. These results indicate that preoperative and intraoperative detection of CAIX expressing tumors, positive resection margins and metastasis might be feasible using this dual labeled antibody preparation. Clinical studies are warranted to assess the safety, feasibility and validity of this imaging approach in patients with ccRCC.

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