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Targeting renal cell carcinoma with radiolabeled antibodies

Stijn Muselaers
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Stijn Muselaers
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About the cover

For centuries, lighthouses have been symbols of hope and safety. As beacons of light, they help sailors navigate along treacherous coastlines by marking hazardous places such as cliffs, shoals and reefs. Although the ionizing radiation used in the studies presented in this thesis is not visible to the human eye, molecular imaging of renal cell carcinoma works in the same way. By targeting the malignant cells with specific radiolabeled antibodies, a beacon of ‘light’ is set up at the site of the tumor lesions, making the danger visible. By better knowing all the treacherous sites, patients and doctors are better prepared to continue their challenging journey together.
Targeting renal cell carcinoma with radiolabeled antibodies

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Voor mijn ouders
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Chapter 1

Introduction and outline of the thesis
Introduction

Renal cell carcinomas (RCCs) are malignant renal tumors that originate from the renal cortex and represent the vast majority of primary renal neoplasms (approximately 85%). Other, less frequent malignant renal tumors are transitional cell carcinomas (8%) and nephroblastomas (6%), of which the latter only affects children [1]. From the mid-seventies until the nineties, the incidence of RCCs has steadily increased. Currently, RCC accounts for approximately 2% of all adult malignancies in the Western countries [2]. Approximately 2,200 people are diagnosed with RCC and 950 people succumb to this disease in the Netherlands each year [3]. The rapid increase of this disease in the final quarter of the twentieth century is mainly caused by improved detection with conventional radiological techniques such as ultrasound, Computed Tomography (CT) and Magnetic Resonance Imaging (MRI), but is also associated with the increase of cigarette smoking, obesity, and hypertension [4-6].

RCC is not a single entity, but rather a collection of different types of tumors, each derived from the various parts of the nephron, the principle element of the kidney. They are all characterized by distinct molecular changes, histological features, and clinical phenotypes. The most commonly used pathologic classification is based on the morphology, growth pattern, cell of origin, histological, and molecular basis of the different types [7]. As depicted in Table 1, the clear cell subtype (ccRCC) is most common and accounts for approximately 85% of all RCC cases. ccRCC is known to be one of the most aggressive types of RCC and is associated with significantly poorer survival rates [8, 9]. Moreover, clinical management of this disease is complicated by the fact that this subtype is highly resistant to chemotherapy and radiation [10-13].
Table 1. Incidence of renal cell carcinomas [1, 7]

<table>
<thead>
<tr>
<th>RCC subtype</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clear cell renal cell carcinoma</td>
<td>80-85</td>
</tr>
<tr>
<td>Papillary renal cell carcinoma</td>
<td>11</td>
</tr>
<tr>
<td>Chromophobe renal cell carcinoma</td>
<td>4</td>
</tr>
<tr>
<td>Carcinoma of the collecting ducts of Bellini</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Renal medulary carcinoma</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Xp11 translocation carcinomas</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Carcinoma associated with neuroblastoma</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Mucinous tubular and spindle cell carcinoma</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Renal cell carcinoma unclassified</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

**Current treatment of ccRCC**

The treatment of ccRCC is heavily dependent on disease stage. Surgical resection is usually the first choice in case of localized ccRCC. However, approximately 30% of patients present with metastatic disease, and recurrence occurs in about 40% of patients treated for a localized tumor [14, 15]. Because of the large number of patients with advanced disease and the lack of good response to chemotherapy and radiotherapy [10, 13] the search for new therapeutic strategies has been ongoing since the mid-eighties, resulting in various immunotherapeutic strategies like high dose interleukin-2 (IL-2) and interferon-α (INF-α). Unfortunately, these agents have a significant toxicity profile and clinical benefit was observed in only very few cases [13, 16].

The development of agents targeting the vascular endothelial growth factor (VEGF) pathway, tyrosine kinase inhibitors (TKIs) and mammalian target of rapamycin inhibitors (mTORs) mark a new era in the treatment of metastatic ccRCC in terms of progression-free survival (PFS) [17-25]. The choice for a specific agent is often complex and depends on the prognostic score according to the Memorial Sloan-Kettering Cancer Center (MSKCC) scoring system, extent of
Introduction

the disease, location of metastases, and any prior treatment. Table 2 provides a brief overview of the current first-line therapeutic options of metastatic ccRCC.

Although targeted agents result in clinical benefit in the majority of patients, side effects like hypertension, fatigue, nausea, hand-foot skin reactions, and diarrhea are frequently seen and can be severe, sometimes even leading to dose reduction. In addition, little is known about the long term effects of these agents [26]. Moreover, treatment with these agents is chronic and cessation of treatment can lead to flare-up of the disease [27]. Finally, because eventually treatment resistance occurs in almost all patients, ccRCC remains a largely incurable disease. Because of these unmet needs in the treatment of ccRCC, the search for novel systemic treatment strategies with less toxicity and significant anti-tumor effect has continued.

Table 2. Current first-line treatment strategies for metastatic RCC. Adapted from the EAU guidelines on renal cell carcinoma [41]

<table>
<thead>
<tr>
<th>RCC subtype</th>
<th>Prognostic group</th>
<th>First-line</th>
</tr>
</thead>
<tbody>
<tr>
<td>ccRCC</td>
<td>Good</td>
<td>sunitinib</td>
</tr>
<tr>
<td></td>
<td>or</td>
<td>pazopanib</td>
</tr>
<tr>
<td></td>
<td>intermediate</td>
<td>bevacizumab + INF-α</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>INF-α</td>
</tr>
<tr>
<td>Non-clear cell RCC</td>
<td>Any</td>
<td>No consensus</td>
</tr>
</tbody>
</table>
Tumor-associated antigens

An alternative therapeutic approach in cancer treatment is to exploit tumor-associated antigens (TAAs) expressed by tumor cells. TAAs are present on both malignant cells and normal cells, but the expression of these antigens is significantly higher on tumor cells. A variety of molecules can be used to target TAAs, such as peptides or monoclonal antibodies (mAb). By coupling chemotherapeutic agents, toxins or radionuclides to these targeting molecules, a cytotoxic load can be guided specifically to the tumor.

Targeting Carbonic Anhydrase IX with monoclonal antibody G250

Over the years, several mAbs against TAAs have been defined in RCC [28-33]. Most of these mAbs only detect certain subtypes of RCC and show extensive crossreactivity with non-tumorous tissues. The murine mAb G250 that recognizes a TAA on RCC tumor cells was first described in 1986 [34]. The then named ‘G250-antigen’ was later identified as Carbonic Anhydrase isoform IX (CAIX), a protein that plays an important role in maintaining the intracellular pH under hypoxic conditions [35, 36].

In normoxic conditions, hypoxia-inducible-factor-1α (HIF-1α) binds to the Von Hippel Lindau protein (pVHL) and is subsequently rapidly degraded. Reduced oxygen availability leads to inhibition of binding of pVHL to HIF-1α, resulting in downstream transcription of hypoxia-inducible genes such as VEGF, platelet-derived growth factor (PDGF) and Carbonic Anhydrase IX (CAIX). VEGF and PDGF both play an important role in angiogenesis, whereas CAIX helps regulating the intra- and extracellular pH and fluid balance by catalyzing the reversible reaction $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+$ [35, 36]. In several solid tumor types, spatial disorganization and flow-based disruption of an abnormal microvasculature is initiated by the growing tumor. These processes lead to hypoxia and ultimately to upregulation of hypoxia-inducible genes, including CAIX [37].
In ccRCC, hypoxia is mimicked by a molecular defect in one of the key proteins in the cascade, namely pVHL. Due to a mutation in the VHL gene and the subsequent (functional) loss of pVHL, the binding of this protein to HIF-1α does not occur. Because of the constitutive presence of HIF-1α in ccRCC, the downstream hypoxia-inducible genes are upregulated, ultimately leading to VEGF, PDGF and CAIX overexpression in these tumors. Other types of RCCs (and other carcinomas) demonstrate limited expression of CAIX, which can be attributed to local hypoxia [37]. However, ccRCC lesions more often and more consistently demonstrate high expression of the antigen [38, 39]. CAIX is not found in normal renal tissue nor in benign cysts, whereas high expression is reported in up to 94% of the ccRCC cases [39, 40]. Only low levels are expressed in other organs, mainly in the upper gastrointestinal tract and the bile ducts [40].

In view of the high and specific CAIX expression in ccRCC, it is an excellent TAA that can be targeted with mAb G250 for both imaging and treatment of ccRCC lesions. Although G250 is not the only antibody that targets CAIX, it is the most extensively investigated one, and the only one that has been used in clinical trials to date. While the first clinical studies performed with radiolabeled murine G250 were highly promising, the murine antibody provoked human anti-mouse antibodies (HAMA) responses in all patients. To circumvent this HAMA-formation, a chimeric (murine/human) variant of G250 was produced, which was later denominated girentuximab.
Chapter 1

Thesis outline

The aim of this thesis was to further optimize the detection and treatment of ccRCC using radiolabeled mAb cG250/girentuximab. The preclinical and clinical studies presented in this thesis focus on detection of ccRCC with Indium-111 (\(^{111}\text{In}\)) labeled girentuximab and treatment of advanced disease with Lutetium-177 (\(^{177}\text{Lu}\))-girentuximab. In addition, the feasibility of novel fluorescence imaging techniques for ccRCC which may ultimately lead to intraoperative detection of ccRCC lesions was evaluated.

Chapter 2 provides an overview of the literature on previous efforts to develop successful strategies for both the detection and treatment of metastatic ccRCC using mAb G250/girentuximab.

Chapter 3 describes the use of \(^{111}\text{In}\)-girentuximab immunoSPECT in patients with lesions suspect for ccRCC. Patients with renal masses of unknown origin and patients with a history of ccRCC with lesions suspect for metastases on follow-up CT scanning were included in this report.

In chapter 4, a novel targeted fluorescence imaging technique was tested. These experiments helped studying the potential to detect ccRCC tumors with fluorescence imaging using mAb girentuximab conjugated with IRDye800CW.

To evaluate targeted fluorescence imaging in a model that better mimics the clinical situation, combined radionuclide and fluorescence imaging was tested by injecting dual-labeled antibody preparation \(^{111}\text{In}\)-G250-IRDye800CW in mice with small, intraperitoneally growing ccRCC lesions. Chapter 5 presents the results of this study.
In chapter 6, the potential of radioimmunodetection and RIT with radiolabeled G250 was evaluated in an advanced xenograft model with intraperitoneally growing ccRCC lesions. After optimizing the antibody dose, a RIT study was performed to determine the efficacy of a single injection of $^{177}$Lu-G250 in this model.

An important challenge to successfully implement girentuximab-based RIT in patient care is optimizing the combination of this therapeutic approach with the current standard of care in metastatic ccRCC, namely treatment with tyrosine kinase inhibitors. Chapter 7 describes a study investigating the effect of neo-adjuvant treatment with tyrosine kinase inhibitor sorafenib on the uptake of mAb girentuximab.

Chapter 8 presents the preliminary results of an ongoing phase II RIT trial. In this trial, patients with advanced metastatic ccRCC are treated with lutetium-177 labeled girentuximab at the maximum tolerated dose level. Both preliminary therapeutic efficacy as well as side effects are described.
References


Chapter 2

Molecular imaging and Carbonic Anhydrase IX-targeted radioimmunotherapy in clear cell renal cell carcinoma

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\textit{Immunotherapy} 2013 May;5(5):489-95
Summary

Conventional imaging is suboptimal at evaluating disease status in renal cell carcinoma (RCC) because of poor sensitivity. Furthermore, there is an unmet need for the treatment of metastatic RCC, both in terms of improvement of progression-free survival and limitation of toxicity. For this reason, radionuclide imaging and radionuclide therapy are extensively investigated. This review provides an overview of the current progress in molecular imaging and radionuclide therapy in clear cell RCC (ccRCC) and will focus on promising detection and therapy strategies targeting the Carbonic Anhydrase IX antigen, which is expressed in ccRCC.
Introduction

Renal cell carcinoma (RCC) accounts for approximately 2% of the total cancer incidence. The worldwide mortality of this disease exceeds 100,000 per year and has been stable for the last few years [1–3]. Surgical resection is usually the first choice in case of localized disease, but approximately 30% of patients with RCC present with metastatic disease and recurrence occurs in approximately 40% of patients treated for a localized tumor. Unfortunately, conventional imaging studies cannot reliably distinguish benign solid lesions from RCC, which frequently poses a diagnostic problem for clinicians. Ultrasound or computed tomography (CT)-guided biopsies have a relatively high sensitivity and specificity [6,7], but are invasive and require careful observation of the patient after the procedure. Adequate characterization of suspect lesions based on imaging is essential to avoid invasive biopsies and superfluous surgery, both in localized and advanced disease.

Besides the clear diagnostic issues, there is an unmet need for improved treatment of this disease. Because of the large number of patients with advanced disease and the lack of a good response to chemotherapy [8,9], the search for therapeutic options has been progressively extended since the mid 1990s, resulting in various immunotherapeutic strategies, such as high-dose IL-2 and IFN-α, albeit with moderate success in few cases [9,10]. The development of agents targeting the VEGF pathway, such as bevacizumab (with IFN-α), tyrosine kinase inhibitors (TKIs), such as sunitinib, pazopanib, axitinib and sorafenib, and mTOR inhibitors, such as temsirolimus and everolimus, mark a new era in the treatment of metastatic RCC in terms of progression-free survival [11–19], but with several disadvantages. First, side effects, such as hypertension, fatigue, nausea, hand–foot skin reactions and diarrhea, are frequently seen and little is known regarding the long-term effects of these agents [20]. Second, these agents have a cytostatic effect on advanced disease, rather than a cytoreductive effect. As a result, treatment with
TKIs and mTOR inhibitors is chronic and cessation of treatment may lead to flare-up of the disease [21]. The search for novel systemic treatment strategies with less toxicity and significant antitumor effects has led to therapeutic regimens using radiolabeled antibodies specifically targeting tumor-associated antigens expressed on tumor cells. This review summarizes the current knowledge on radionuclide imaging of RCC, as well as the experience gained in radioimmunotherapy (RIT) trials in these patients.

**Current molecular imaging modalities in clear cell RCC**

Fluorine-18 ($^{18}$F)-fluorodeoxyglucose (FDG)-PET has been widely investigated and is now an established molecular imaging modality for various malignancies. In RCC, the diagnostic accuracy depends on the tumor grade and the FDG uptake being higher in dedifferentiated tumors, which is also observed in other tumor types. One of the major drawbacks of the detection of RCC with FDG-PET is the excretion of FDG via the kidneys, resulting in relatively high background activity. This can only be partly reduced by hyperhydration or administration of diuretics. Another disadvantage of imaging renal masses with FDG-PET is the reported false-positive signal in patients with angiomyolipoma, pericytoma andpheochromocytoma [22]. Wang et al. recently performed a meta-analysis of 14 articles on the role of FDG-PET and FDG-PET/CT in both primary renal tumors and extrarenal lesions [23]. For primary renal tumors, the pooled sensitivity and specificity of FDG-PET was 62 and 88%, respectively. For detecting metastases of RCC, the pooled patient-based sensitivity and specificity of FDG-PET was 79 and 90%, respectively. The use of FDG-PET/CT improved the accuracy of detecting metastases as the pooled sensitivity and specificity increased to 91 and 88%, respectively. The authors conclude that combining FDG-PET and CT is mainly helpful in detecting extrarenal metastasis rather than renal lesions. However, the total number of studies assessing the role of FDG-PET/CT evaluated in this meta-analysis is limited and further studies are warranted.
Other PET tracers, such as $^{18}$F-thymidine [24], $^{18}$F-fluoromisonidazole [25] and carbon-11-acetate [26], were also investigated in patients with RCC and, although reasonably good accuracy has been reported, the exact role of these tracers has not yet been studied in larger trials.

**Carbonic Anhydrase IX and girentuximab**

In 1986, Oosterwijk et al. described a monoclonal antibody (G250) that targeted an antigen highly expressed in clear cell RCC (ccRCC), the most common renal malignancy found in approximately 80–85% of cases [27]. A role as a carrier for the treatment of ccRCC was suggested. Later, a chimeric version of the monoclonal antibody G250 (cG250) was constructed and designated girentuximab [28]. The antigen was later recognized as a member of the Carbonic Anhydrase (CA) family and has been denominated MN/CA9/CAIX in the literature [29,30]. The CAs form a family of enzymes that catalyze the reversible reaction $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+$, making these enzymes key players in the regulation of intra- and extra-cellular pH, and fluid balance. CAIX is ubiquitously expressed in ccRCC and not in normal renal tissue. Other types of RCCs, such as papillary RCCs, unclassified RCCs and Xp11.2 translocation RCCs, also have some membranous expression of CAIX however, ccRCCs more often and more consistently demonstrated high (up to 94%) expression compared with any other tumor type [31,32]. An extensive study by Leibovich et al. showed that CAIX is also expressed at low levels in the gastric mucosa, pancreatobiliary epithelium, small intestine crypt base, mesothelial cells, ovarian surface epithelium and fetal rete testis, but was not found in the normal kidney [33]. Many studies have focused on the potential of CAIX to predict clinical outcome, but no consensus has been reached [32–36], although high levels of CAIX seem to correlate with a better response to IL-2 therapy [37].

Because of the specific expression of CAIX on primary, as well as metastatic, ccRCC
tumors, the antigen is considered to be an excellent target for imaging of both localized and advanced disease. To date, several thousand girentuximab injections have been performed worldwide in several clinical trials since its introduction, and no severe side effects or allergic reactions to the antibody infusions performed in these studies were reported (for detailed toxicity data see [38,39]).

**CAIX-mediated radioimunodetection**

Several clinical trials have demonstrated the possibility of detecting CAIX-expressing ccRCC lesions using girentuximab labeled with iodone-131 ($^{131}$I), iodone-124 ($^{124}$I) or indium-111 ($^{111}$In). In a phase I protein dose-escalation study to determine the pharmacokinetics, toxicity, immunogenicity and imaging characteristics of $^{131}$I-girentuximab, clear antibody targeting was observed in patients with CAIX-positive tumors at the optimum protein dose of 5–10 mg [40]. In a head-to-head comparison with FDG-PET, $^{131}$I-labeled girentuximab was found to be inferior at detecting ccRCC metastases [41]. These results led to the search for alternative radionuclides with more suitable characteristics to improve scintigraphic imaging. In an intrapatient comparative study, labeling girentuximab with the radiometal $^{111}$In proved to be superior to $^{131}$I in terms of imaging characteristics and increased tumor:blood ratios. With the $^{111}$In-labeled tracer, 47 lesions were detected, compared with 30 lesions with the $^{131}$I-labeled tracer [42]. In a larger clinical study, $^{111}$In-girentuximab proved to be a helpful diagnostic tool in both localized and metastatic ccRCC [43]. In addition to trials with different single-photon emission computed tomography (SPECT) tracers, the PET tracer $^{124}$I-girentuximab was extensively investigated to preoperatively visualize the presence of ccRCC. In a phase I trial in 26 patients scheduled for nephrectomy, 15 out of a total of 16 ccRCC lesions were detected, resulting in a sensitivity of 94%. Because of the high specificity (100%; 95% CI: 66–100), and negative (90%; 95% CI: 55–100) and positive (100%; 95% CI: 78–100) predictive values, it was concluded that $^{124}$I-girentuximab
immunoPET can identify ccRCC accurately, and help in clinical decision-making when dealing with renal masses of uncertain origin [44]. A subsequent study by Pryma et al. found a significant correlation between the PET measurements and autoradiography of the surgical specimens of the aforementioned clinical trials, suggesting immunoPET may be useful in quantitatively assessing antigen targeting by antibody-based therapies [45]. Recently, a large multicenter study comparing the diagnostic accuracy of $^{124}\text{I}$-girentuximab PET/CT with that of diagnostic CT for the detection of ccRCC in presurgical patients with renal masses was completed. Of the 226 patients enrolled, 204 were infused with 185 MBq $^{124}\text{I}$-girentuximab 2–6 days prior to image acquisition and, eventually, 195 patients were evaluated. CT scanning was performed within 48 hours of $^{124}\text{I}$-girentuximab PET/CT. This study confirmed the high accuracy of $^{124}\text{I}$-girentuximab as the reported sensitivity (86%; 95% CI: 75–97) and specificity (86%; 95% CI: 69–100) were markedly higher than those of conventional CT [46,47].

In general, due to the better spatial resolution of PET compared with SPECT, smaller lesions can be visualized and a more exact localization of lesions can be expected with PET. However, the performance of $^{111}\text{In}$-girentuximab and that of $^{124}\text{I}$-girentuximab has not been directly compared to date. Animal data suggest better delineation of lesions is possible with zirconium-89 ($^{89}\text{Zr}$)-labeled girentuximab immunoPET compared with $^{111}\text{In}$-girentuximab immunoSPECT [48].

Whether girentuximab imaging is also helpful in evaluating treatment response of TKIs is currently subject to investigation [49]. In a recently completed clinical trial, a markedly decreased uptake of $^{111}\text{In}$-girentuximab after treatment with the TKI sorafenib was found [50,51]. There was no indication that sorafenib treatment affected CAIX expression. Therefore, this effect is most likely caused by the destruction of the tumor vasculature, resulting in reduced delivery and tumor penetration of the antibody. Although no data have been published regarding the effect of other TKIs in humans,
a similar effect can be expected with sunitinib and pazopanib as these agents have the same mode of action. Limited data from animal experiments suggest increased antibody uptake after discontinuation of sunitinib treatment, presumably due to rapid rebound neovascularization [21].

**RIT in ccRCC**

Extensive research on the development of RIT has yielded a few major breakthroughs, mainly in the treatment of malignant non-Hodgkin's lymphoma with the approved $^{90}$Y-ibritumomab tiuxetan (Zevalin®; Spectrum Pharmaceuticals, Inc., NV, USA) and $^{131}$I-tositumomab (Bexxar®; GlaxoSmithKline, London, UK) [52-55]. RIT has also been studied in a number of solid tumors, albeit less successful in terms of clinical response. The observation that girentuximab could specifically target ccRCC lesions or guide radionuclides to malignant lesions has stimulated studies on the development of antibody-mediated therapy and RIT for ccRCC. Several clinical trials were conducted with the unlabeled ‘cold’ antibody girentuximab as an adjuvant therapy after nephrectomy. Although data are yet to be published, it has been reported that the large, recently completed phase III ARISER trial [56] did not meet its primary endpoint of median disease-free survival [57]. Currently, subanalyses of the degree of CAIX expression and clinical outcome are ongoing.

The first RIT trial was conducted to determine targeting and toxicity of $^{131}$I-labeled murine G250. A total of 33 patients with metastatic ccRCC were treated with escalating doses ranging from 555 to 3330 MBq/m$^2$ of $^{131}$I-labeled G250. Three of the 15 patients treated at the maximum tolerated dose (MTD) of 3330 MBq/m$^2$ showed stable disease, which lasted up to 18 weeks post-treatment. The major drawback of the use of murine G250 was the formation of human antimouse antibodies in all patients after injection, restricting therapy to a single infusion [58]. The first clinical trial using girentuximab
labeled with $^{131}\text{I}$ was a phase I dose-escalation study. In this trial, all patients ($n = 12$) received a diagnostic infusion of 222 MBq $^{131}\text{I}$-girentuximab. If targeting of the antibody in metastases was observed, a therapeutic infusion with girentuximab labeled with a high dose of $^{131}\text{I}$ followed. Besides a partial response in one patient, no objective responses were seen. Because grade IV hematological toxicity in the two patients who received a dose of 2775 MBq/m$^2$ was observed, the MTD was set at 2220 MBq/m$^2$ [58]. In an attempt to limit the hematological toxicity of $^{131}\text{I}$-girentuximab RIT, a fractionated-dose study was initiated, in which patients received an initial dose of 1110 MBq $^{131}\text{I}$-girentuximab. Whole-body activity was measured after 2–3 days and a second administration of $^{131}\text{I}$-girentuximab was administered to, again, reach a total of 1110 MBq of radioactivity in the body. This procedure was repeated until a whole-body absorbed dose of 0.50 Gy was reached. Patients without disease progression were re-treated after recovery from hematological toxicity. In subsequent cohorts, the whole-body absorbed dose was increased by 0.25 Gy. A total of 15 patients were included in the trial. Despite disease stabilization in seven patients, no major clinical responses were seen. Formation of human antichimeric antibodies (HACA) was seen in two patients, prohibiting them from being treated continually. Although fractionated RIT with $^{131}\text{I}$-girentuximab was safe and feasible, hematological toxicity was not significantly affected by fractionation [60]. Another phase I/II trial evaluated the safety and efficacy of two sequential high-activity dose treatments with $^{131}\text{I}$-girentuximab. As in other trials, a scout dose preceded the therapeutic infusion of $^{131}\text{I}$-girentuximab. The therapeutic dose was at the predetermined MTD of 2220 MBq/m$^2$. Patients with stable disease (or responders) were eligible to receive a second dose at 75% of the MTD of the first RIT cycle 12 weeks later. In the majority of patients, two cycles of $^{131}\text{I}$-girentuximab could be safely administered without severe toxicity. In this study, no objective responses were observed, although two RIT doses resulted in stabilization of previously progressive disease in five out of 27 patients. The formation of HACA after multiple girentuximab administrations was observed in eight out of 27 patients [61].
Dosimetric analysis of the imaging data obtained in this trial revealed that the radiation absorbed dose to the tumor was relatively low in larger lesions, whereas only lesions less than 5 g absorbed more than 50 Gy, suggesting that RIT is particularly suitable for the treatment of small-volume disease or treatment in an adjuvant setting [62].

Due to the limited clinical benefit obtained in the first clinical RIT trials, the search for more suitable radionuclides was initiated. A preclinical study revealed superior therapeutic efficacy of lutetium-177 ($^{177}$Lu), yttrium-90 ($^{90}$Y) or rhenium-186 ($^{186}$Re)-labeled girentuximab compared with $^{131}$I-labeled girentuximab. Tumor growth in mice was delayed most effectively by $^{177}$Lu-girentuximab, resulting in a median survival for the $^{177}$Lu group that was almost twice as long as that for the $^{131}$I group (294 vs 164 days) [63]. This led to various additional animal experiments and ultimately in a phase I dose-escalation trial with $^{177}$Lu-girentuximab. Because the in vivo characteristics of $^{111}$In-girentuximab are very similar to those of $^{177}$Lu-girentuximab, patients with advanced ccRCC initially received a scout dose of $^{111}$In-girentuximab to evaluate antibody uptake in tumor lesions. If at least one evaluable metastatic lesion <5 cm in diameter was visualized with $^{111}$In-girentuximab, $^{177}$Lu-girentuximab was administered at escalating doses from 1110 to 2590 MBq/m$^2$ in cohorts of three patients per dose level. Approximately 70% of the patients showed stable disease after one treatment cycle and one patient had a partial response lasting up to 9 months. A total of 13 patients received two treatment cycles and four of them received three treatment cycles. In two patients, HACA formation was detected [64]. Currently, the subsequent phase II trial to determine the efficacy of multiple doses of $^{177}$Lu-girentuximab at the MTD of 2405 MBq/m$^2$ is ongoing [65]. If an objective response (at least stable disease) is seen after the first treatment cycle, patients are eligible for a maximum of two additional treatment cycles at 75% of the activity dose of the previous treatment cycle [65].

Another ongoing clinical trial is a phase I activity dose-escalation study using
Similar to the \textsuperscript{177}Lu-girentuximab trial, patients first receive a scout dose of \textsuperscript{111}In-girentuximab and if there is evidence of targeting to lesions, \textsuperscript{90}Y-girentuximab is administered. At least three patients per dose level will be followed for up to 8 weeks. The dose levels start at 7.4 MBq/kg \textsuperscript{90}Y and subsequent groups will be treated in 3.7 MBq/kg increments, with the last cohort increasing by 1.85 MBq/kg.

In contrast to the previously mentioned phase I/II trial \cite{64,65}, patients will receive only one treatment. In both ongoing trials, a series of SPECT acquisitions is performed after administration of the scout dose, providing data for additional dosimetric studies. As postulated by several groups, individualized image-based dosimetry is probably required for the optimal therapeutic delivery of radiolabeled antibodies \cite{67,68}.

**Conclusion**

Conventional imaging with CT, MRI and FDG-PET is suboptimal in evaluating disease status in RCC due to its poor sensitivity. Radionuclide imaging has proven to be a useful tool in detection of localized and metastatic disease. Despite the recent emergence of angiogenesis inhibitors, there is still an unmet need to effectively treat metastatic RCC. Early reports of radioimmunotherapy targeting the CAIX antigen in ccRCC indicate great potential of this new treatment strategy, but further studies are needed to confirm these results.
Future perspective

As a recent large, phase III clinical trial showed no therapeutic efficacy of unlabeled girentuximab in the adjuvant setting [56], future studies are more likely to focus on radioimmunodetection and RIT with radiolabeled girentuximab. Radioimmunoscintigraphy with radiolabeled girentuximab holds great promise for the future, both in detecting localized and advanced disease. However, based on current data, it is not possible to conclude which radiotracer is superior in diagnostic performance. In the near future, a clinical trial with the PET tracer $^{89}$Zr-girentuximab will be initiated in our center, which will provide additional information regarding the use of girentuximab-based immunoPET in ccRCC. Besides the usefulness in radioimmunodetection, girentuximab is a potent carrier for RIT in ccRCC. However, there are still several hurdles to overcome before girentuximab-based RIT can be implemented as a standard treatment. As previously mentioned, it is not clear which patients benefit most from RIT. Past results indicate that RIT is mainly suitable for the treatment of small-volume disease or possibly as adjuvant treatment in selected cases, and more evidence regarding this topic is expected from the ongoing clinical trials with $^{90}$Y and $^{177}$Lu-labeled girentuximab in the upcoming years [62]. Besides better patient selection in the future, advances in dosimetric analysis will presumably contribute to the improvement of RIT as the trade-off between efficacy and toxicity can be better tailored to the individual patient. Lastly, an important deficit in our current knowledge is how to optimally combine girentuximab-based RIT with the current standard of care in metastatic ccRCC. As described earlier, we recently found a markedly decreased uptake of $^{111}$In-girentuximab after treatment with the TKI sorafenib [50,51]. Data from this study suggest that the effect of girentuximab-based RIT would be severely hampered if given during TKI treatment. Further studies to evaluate the duration of this TKI-induced effect are needed and may prove to be a vital step to successfully combine TKI treatment and RIT for ccRCC patients.
References


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Chapter 3

Indium-111 labeled girentuximab immunoSPECT as a diagnostic tool in clear cell renal cell carcinoma


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Abstract

Background

Improved and more frequent radiologic evaluation has resulted in increased identification of renal masses of unknown origin, which frequently pose a diagnostic dilemma for urologists.

Objective

Carbonic Anhydrase IX (CAIX) is an antigen ubiquitously expressed in clear cell renal cell carcinoma (ccRCC). The specific and high level of expression in ccRCC makes CAIX an excellent target for imaging ccRCC lesions. We present our experience with immuno–single-photon emission computed tomography (immunoSPECT) imaging with the indium-111 ($^{111}$In)–labeled anti-CAIX antibody girentuximab in patients presenting with either a primary renal tumor or a history of ccRCC and lesions suspect for metastases during follow-up.

Design, setting, and participants

Twenty-nine patients received 100–200 MBq $^{111}$In-labeled girentuximab. Whole-body and single photon emission computed tomography (SPECT) images were acquired after 4–7 days.

Intervention

Injection with $^{111}$In-girentuximab and image acquisition after 4–7 days.
Outcome measurements and statistical analysis

Accuracy of $^{111}$In-girentuximab immunoSPECT.

Results and limitations

Distinct uptake of $^{111}$In-girentuximab was seen in 16 of 22 patients presenting with a renal mass. All renal masses proven to be ccRCC after resection ($n = 15$) were detected with $^{111}$In-girentuximab. Suspect lesions of six patients showed no uptake of $^{111}$In-girentuximab. In these patients, ccRCC was not found, nor progression occurred. Seven patients with a history of ccRCC and possible metastatic lesions on follow-up computed tomography scans were imaged with $^{111}$In-girentuximab. In four of these patients, the lesions showed preferential uptake of $^{111}$In-girentuximab and local or systemic treatment was initiated. In three other cases, no $^{111}$In-girentuximab targeting was seen. During follow-up of these three patients, one showed progression, for which systemic treatment was started. In the two other patients, no progression occurred, suggesting a benign nature.

Conclusions

$^{111}$In-girentuximab immunoSPECT can be used to detect ccRCC lesions in patients with a primary renal mass and to clarify the nature of lesions suspect for metastases in patients with a history of ccRCC.
Introduction

Improved and more frequent radiologic evaluation has resulted in increased identification of small renal masses of unknown origin. Unfortunately, conventional imaging studies cannot reliably distinguish benign solid lesions from renal cell carcinoma (RCC). This frequently poses the dilemma for urologists of whether to perform a (partial) nephrectomy on a potentially benign mass or to refrain from surgery and enter patients with a potentially aggressive malignancy into a follow-up protocol until disease progression occurs. Specimens obtained by ultrasound- or computed tomography (CT)–guided biopsies have a relatively high sensitivity and specificity [1,2], but the procedures are invasive and require careful observation of the patient afterward. In addition, a recent report found a pooled sensitivity of fluorine-18 fluorodeoxyglucose positron-emission tomography ($^{18}$F-FDG-PET) imaging for detecting primary RCC of only 62% [3]. Adequate characterization of these renal lesions based on imaging would be advantageous to avoid invasive biopsies and, more importantly, superfluous surgery. Similarly, unambiguous detection of lesions suspect for metastatic clear cell RCC (ccRCC) would be advantageous to start additional treatment.

Carbonic Anhydrase IX (CAIX) is a specific antigen for ccRCC [4,5]. CAIX catalyzes the reversible reaction $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+$ and helps regulate the intra- and extracellular pH and fluid balance in the otherwise highly acid environment of the malignant cells. The antigen is not found in normal renal tissue nor in benign cysts, whereas high levels of expression are reported in up to 94% of the ccRCC cases [4,5]. Only very low levels are expressed in other organs, mainly in the upper gastrointestinal tract [5]. Because of its specific and high level of expression, CAIX is an excellent target for imaging ccRCC lesions. In 1986, the monoclonal antibody (mAb) girentuximab (G250) that recognized an antigen on RCC tumor cells was described [6]. The unknown antigen was later identified
as a member of the Carbonic Anhydrase family and has been denominated MN/CA9/CAIX in literature [7,8]. To reduce the immunogenicity of the murine mAb, a chimeric version of G250 was constructed [9], which was denominated girentuximab. More than 2500 girentuximab injections have been performed worldwide in several clinical trials since its introduction [10-17]. No severe side effects or allergic reactions to the antibody infusions have been reported to date. In 2007, Divgi et al. reported on the possibility of specifically detecting ccRCC preoperatively with positron-emission tomography (PET) using iodine-124 \(^{124}\text{I}\)-labeled girentuximab in patients with renal masses [16]. In our center, girentuximab labeled with the gamma-emitting radionuclide indium-111 \(^{111}\text{In}\) is used to detect ccRCC lesions. Single-photon emission CT (SPECT) with \(^{111}\text{In}\)-labeled girentuximab has several important advantages. Similar to \(^{124}\text{I}\)-girentuximab immunoPET, it is noninvasive and does not require the use of intravenous contrast agents, which makes it suitable for patients with an impaired renal function. In addition, it is easier to produce as an off-the-shelf agent than \(^{124}\text{I}\)-girentuximab, which requires specialized equipment and needs to be purified after the labeling procedure.

In this paper, we present our experience with \(^{111}\text{In}\)-girentuximab immunoSPECT in patients presenting with a primary renal tumor or having a history of ccRCC with lesions on follow-up imaging suspect for metastases.

**Materials and methods**

**Radiolabeling of girentuximab**

Girentuximab (cG250; Wilex AG, Munich, Germany) was conjugated with diethylenetriaminepentaacetic acid (DTPA) as described previously [18]. The girentuximab–DTPA solution was aliquoted into sterile vials (5 mg in 1.0 ml) and stored at −20 °C until use. After inclusion of a patient, the girentuximab-DTPA
conjugate was labeled with 100–200 MBq of $^{111}$In (Covidien, Petten, The Netherlands). The volume of $^{111}$In-DTPA–girentuximab was adjusted to 10 ml with normal saline. Radiochemical purity of all preparations used in this study exceeded 95%, as determined by instant thin-layer chromatography.

**Study design**

In total, $^{111}$In-girentuximab immunoSPECT data from 29 patients were analyzed. Twenty-two patients with an incidentaloma were included, of whom 15 patients participated in a molecular imaging trial (NCT00602862) [19]. In addition, $^{111}$In-girentuximab imaging data from seven patients with a history of localized ccRCC and who presented with lesions suspect for metastases were analyzed. Informed consent was obtained from all patients. $^{111}$In-girentuximab was infused as a slow bolus. After administration, patients were observed for 30 minutes. The dose was 100 MBq $^{111}$In for patients in the molecular imaging trial, and 185–200 MBq $^{111}$In for all other patients. Whole-body and SPECT images were acquired 4–7 day after injection.

**Imaging**

Whole-body scans were acquired 4–7 days after injection using a dual head $\gamma$-camera (E.cam or Symbia T16; Siemens Inc, Munich, Germany) equipped with parallel-hole medium-energy collimators (symmetric 15% window over 172 and 247 keV) and a scan speed of 4 cm/min [10]. Subsequently, three-dimensional (3D) SPECT of the area of interest was acquired. All images were reviewed by nuclear medicine physicians and discussed in multidisciplinary tumor boards.
<table>
<thead>
<tr>
<th>No.</th>
<th>Sex</th>
<th>Age (y)</th>
<th>Indication for scintigraphy</th>
<th>Size of lesion(s)</th>
<th>Targeting</th>
<th>Type</th>
</tr>
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<td>Renal mass</td>
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<td>+</td>
<td>ccRCC</td>
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<td>ccRCC</td>
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<td>M</td>
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<td>Renal mass and lung lesions</td>
<td>Ø 7.9 cm, largest lung lesion 3.1 cm</td>
<td>+,+</td>
<td>ccRCC</td>
</tr>
<tr>
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<td>F</td>
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<td>Ø 6.4 cm</td>
<td>+</td>
<td>ccRCC</td>
</tr>
<tr>
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<td>M</td>
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<tr>
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<td>Oncocytoma</td>
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<td>F</td>
<td>69.8</td>
<td>Renal mass</td>
<td>Ø 8.5 cm</td>
<td>+</td>
<td>ccRCC</td>
</tr>
<tr>
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<td>+</td>
<td>ccRCC</td>
</tr>
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<td>M</td>
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<td>Renal mass</td>
<td>Ø 2.5 cm</td>
<td>-</td>
<td>Oncocytoma</td>
</tr>
<tr>
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<td>Ø 4.9 cm</td>
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<td>ccRCC</td>
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<td>MTSCC, lung: chondrohamartoma</td>
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<td>F</td>
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<td>+</td>
<td>ccRCC</td>
</tr>
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<td>45.5</td>
<td>History of ccRCC, abdominal lesions</td>
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<td>+,+</td>
<td>ccRCC</td>
</tr>
<tr>
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<td>63.6</td>
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<td>Multiple small lesions</td>
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<td>No histopathology</td>
</tr>
<tr>
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<td>F</td>
<td>59.8</td>
<td>History of ccRCC, cystic abdominal lesion</td>
<td>Ø 9.0 cm</td>
<td>±</td>
<td>Cystic lesion, small ccRCC component</td>
</tr>
<tr>
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<td>Ø 3.1 cm</td>
<td>-</td>
<td>No histopathology</td>
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<tr>
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<td>M</td>
<td>78.3</td>
<td>History of ccRCC, intra aortacaval lesion</td>
<td>Ø 1.5 cm</td>
<td>±</td>
<td>ccRCC</td>
</tr>
<tr>
<td>28</td>
<td>F</td>
<td>63.7</td>
<td>History of ccRCC, abdominal lesion</td>
<td>Ø 7.5 cm</td>
<td>-</td>
<td>No histopathology</td>
</tr>
<tr>
<td>29</td>
<td>F</td>
<td>59.0</td>
<td>History of ccRCC, liver lesion</td>
<td>Ø 0.5 cm</td>
<td>+</td>
<td>No histopathology</td>
</tr>
</tbody>
</table>

Abbreviations: ccRCC = clear cell renal cell carcinoma, MTSCC = mucinous tubular spindle cell carcinoma

Table 1. Patient and imaging characteristics
Results

Patients

In total, $^{111}\text{In}$-girentuximab immunoSPECT data from 29 patients were analyzed. Patient characteristics and imaging data are summarized in Table 1. Twenty-two patients were scanned because of an incidentaloma of the kidney and seven patients were scanned to detect metastases of ccRCC. $^{111}\text{In}$-girentuximab infusions were well tolerated and no side effects were observed.

Imaging

Renal mass

Of the 22 patients who presented with a renal mass of unknown origin, clear preferential uptake of $^{111}\text{In}$-girentuximab was observed in the lesions of 16 patients. These lesions were resected and histopathologic examination revealed ccRCC in 15 patients, all detected by $^{111}\text{In}$-girentuximab imaging. A typical example of a positive $^{111}\text{In}$-girentuximab scan is shown in Figure 1. In one of 16 patients, $^{111}\text{In}$-girentuximab targeting was found in a type 2 papillary RCC with CAIX expression (Patient 9). The CAIX expression in this particular patient was found in a vital tumor region within a necrotic part of the surgical specimen, suggesting this particular expression could be hypoxia driven (Figure 2). This was the only case in which targeting was seen in a non-clear cell subtype. Overall, in this group of patients, the positive predictive value (PPV) of $^{111}\text{In}$-girentuximab imaging for ccRCC was 94%.

In six patients presenting with a renal mass, no targeting of $^{111}\text{In}$-girentuximab was observed. Four of these patients underwent surgery as planned (Patients 11, 16,
Histopathologic evaluation revealed two cases of benign oncocytoma (Patients 16 and 19), a chromophobe in Patient 11, and a mucinous tubular and spindle cell carcinoma subtype in Patient 21. Patient 21 also presented at diagnosis with a lung lesion, which proved to be a benign chondrohamartoma. Both the renal tumor and the lung lesion were not targeted with $^{111}$In-girentuximab. The remaining two patients (Patients 3 and 15) were closely monitored with repeated CT scans. No growth of the renal mass occurred in the follow-up period (>24 months).

**Figure 1.** Posterior $^{111}$In-girentuximab image of Patient 14 with a small renal mass in the left kidney. An attempt to biopsy the lesion had failed and the patient underwent $^{111}$In-girentuximab immunoSPECT. Besides normal uptake in the right upper abdomen (liver and upper gastrointestinal tract), clear targeting of $^{111}$In-girentuximab is seen in lesion (arrow). Histopathologic examination revealed a clear cell renal cell carcinoma with a 12-mm diameter.

**Figure 2.** Carbonic Anhydrase IX (CAIX) expression in a type 2 papillary tumor. CAIX expression was found in a vital tumor region within a necrotic part of the surgical specimen, suggesting hypoxia-driven expression.
Detection of clear cell renal cell carcinoma metastases

Seven patients with a history of ccRCC and possible metastatic lesions on follow-up CT scans were imaged with $^{111}$In-girentuximab (Table 1). In four patients, positive $^{111}$In-girentuximab imaging was observed. Patient 23 had abdominal masses near the rectus abdominis muscle (36 mm) and between the spleen and diaphragm (16 mm). Targeting of $^{111}$In-girentuximab was seen in both lesions. Both lesions were resected, and both proved to be ccRCC metastases. Patient 25 presented with a large, cystic, abdominal lesion suspect for local recurrence. Focal targeting of $^{111}$In-girentuximab in the large cystic mass was seen. Histopathologic examination revealed a local recurrence of ccRCC. Patient 27 presented with an enlarged para-aortal lymph node of 15 mm 10 years after the initial diagnosis of ccRCC. $^{18}$F-FDG-PET was negative. Moderate targeting of $^{111}$In-girentuximab was detected on the whole-body images, and the patient underwent a retroperitoneal lymph node dissection. Histology showed subtotal lymph node infiltration of CAIX-positive malignant cells. Patient 29 had a liver lesion of 0.5 cm on follow-up CT scan (Figure 3) 6 months after nephrectomy because of ccRCC. The lesion was biopsied, but unfortunately, specimen histology was not conclusive. $^{111}$In-girentuximab imaging was performed 4 weeks later and showed targeting of the antibody in this lesion, strongly suggesting metastatic disease. Follow-up CT scanning 2 months thereafter confirmed disease progression, after which systemic treatment was started (Figure 3). Patient 24 had known metastatic ccRCC and transient interstitial pneumonitis due to interferon-α therapy. $^{111}$In-girentuximab immunoSPECT was performed to assess the nature of multiple, new, pulmonary abnormalities during sunitinib treatment because it was uncertain that these represented active tumor tissue. In contrast to the already known metastases, no targeting in the new lung lesions was seen. Therefore, it was hypothesized that the abnormalities were possibly a flare-up of the interstitial pneumonitis due to the sunitinib treatment, and treatment was discontinued in view of the otherwise stable disease. No progression of these lung
Figure 3. Images of Patient 29, who had a history of clear cell carcinoma. After tumor nephrectomy, the patient entered active surveillance. (A) On follow-up computed tomography (CT) scan, a 5-mm lesion in segment 8 of the liver was detected, and biopsy of this lesion was nonconclusive. (B) Clear targeting of the liver lesion on the subsequent $^{111}$In-girentuximab immunoSPECT was detected. (C) On a follow-up CT scan 2 months after immunoSPECT, growth of the lesion was detected and systemic therapy was started.
lesions occurred for >18 months, suggesting a nonmalignant nature. Patient 26 had a history of localized ccRCC and presented with a slowly growing cystic lesion of 3.1 cm located in the pancreas. Unfortunately, no histology could be obtained because of the localization of the lesion. Scintigraphy showed ambiguous targeting, possibly because of the cystic nature of the lesion. The lesion slowly progressed over time, for which sunitinib treatment was initiated. Patient 28 was referred to our center because of suspicion of local recurrence after radiofrequency ablation (RFA) of a primary ccRCC lesion. A biopsy specimen of the lesion was obtained; however, the histopathologic analysis was inconclusive. In addition, no targeting of $^{111}$In-girentuximab in the abdominal mass was detected. This patient was closely followed with repeated CT scans. No growth of the lesion occurred in a period of >12 months, suggesting the abnormalities seen on CT could be due to the reactive inflammatory response to the RFA treatment.

**Discussion**

In this retrospective analysis of our experience with $^{111}$In-girentuximab immunoSPECT in patients suspected of (recurrent) ccRCC, specific and high levels of radiolabeled antibody targeting was seen in all renal tumors of the ccRCC subtype, as demonstrated by confirmative histopathologic analyses, but not in benign lesions. PPV for detecting primary RCC lesions of the clear cell subtype was very high, as only one of 16 malignant tumors had a different histology. This indicates that if targeting of $^{111}$In-girentuximab is detected in a renal tumor, there is a clear need for surgical intervention. Our observations are in agreement with the clinical study with $^{124}$I-girentuximab immunoPET [16]. This study reported a detection rate of 15 of 16 cases of ccRCC (one negative study in a largely necrotic ccRCC) and absent targeting in 9 of 9 patients with non–ccRCC (including two papillary RCCs). Recently, a large, comparative, multicenter study of $^{124}$I-girentuximab PET/CT versus diagnostic CT for the detection of ccRCC in presurgical patients with renal masses was completed. This study confirmed the high accuracy of $^{124}$I-girentuximab,
as the reported sensitivity (86%; 95% confidence interval [CI], 75–97) and specificity (86%; 95% CI, 69–100) were markedly higher than those of conventional CT [20].

In addition to detection of primary ccRCC, we demonstrated that $^{111}$In-girentuximab imaging can be a helpful, noninvasive tool for clinical decision making in patients with a history of ccRCC who present with lesions suspect for metastases, particularly in cases with inconclusive conventional imaging or biopsy-specimen histology. Although the presence of ccRCC is unlikely in the patients in whom no histopathologic confirmation was obtained, false-negative findings in these particular patients cannot be ruled out completely because targeting of $^{111}$In-girentuximab is dependent on the degree and extent of CAIX expression. Although the vast majority of renal ccRCC lesions do express high levels of CAIX [6,7], inevitably a small subset of these lesions will not be detected due to the absence or low abundance of antigen expressed in the tumor cells. In addition, cystic lesions or subtotally ccRCC-infiltrated lymph nodes can be difficult to detect (eg, Patients 25, 26, and 27) due to the low numbers of CAIX-expressing cells. For solid tumors located in the kidney, a negative $^{111}$In-girentuximab immunoSPECT makes the presence of ccRCC highly unlikely, although another renal malignancy cannot be ruled out. Active surveillance or biopsy in patients with negative $^{111}$In-girentuximab immunoSPECT is warranted to further assess the nature of the suspect lesions.

It remains to be determined whether girentuximab-based radioimmunodetection is also helpful for response evaluation to systemic treatment like tyrosine kinase inhibitors (TKIs). In an imaging study using the $^{111}$In-labeled mAb bevacizumab, a markedly decreased uptake of the antibody in the renal tumors after treatment with the TKI sorafenib was seen. The decreased targeting was most likely due to the destruction of the tumor vasculature, which results in a reduced delivery and accumulation of the antibody in the tumor [21]. The effect of sorafenib on $^{111}$In-girentuximab uptake appears to be very similar [22]. Although no data have been published about the effect of other
TKIs on antibody targeting in humans, a similar effect can be expected with sunitinib and pazopanib, as these agents have the same mode of action. Currently, a clinical trial that focuses on the potential of $^{124}\text{I}$-girentuximab PET to detect early treatment response to sunitinib is ongoing (NCT01582204) [23] and is expected to give more insight to the role of radioimmunodetection in monitoring ccRCC during treatment. Distinct advantages of immunoPET as compared to immunoSPECT are the superior resolution, the shorter duration for full, 3D image acquisition, and the potential for quantitative analysis of tumor targeting. In contrast, the radiation burden after administration of typical radiation doses of $^{124}\text{I}$ and zirconium-89 ($^{89}\text{Zr}$) is markedly higher than that of $^{111}\text{In}$. From the currently available data, we cannot deduce whether a clear difference in the diagnostic performance of immunoSPECT and immunoPET exists. A study with $^{89}\text{Zr}$-girentuximab immunoPET will be initiated in 2013 and will hopefully clarify this matter.

**Conclusions**

$^{111}\text{In}$-girentuximab immunoSPECT is a helpful, noninvasive tool to further characterize incidentalomas of the kidney. Where there is targeting of the tumor, surgical intervention is warranted. In addition, it can be used to clarify the nature of lesions suspect for metastases in patients with a history of ccRCC.
References


Chapter 4

Optical imaging of renal cell carcinoma using the anti-CAIX monoclonal antibody girentuximab

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Abstract

Near-infrared dye-tagged antibodies can be used for sensitive detection of tumor tissue in vivo. Surgery for clear cell renal cell carcinoma (ccRCC) might benefit from optical imaging by facilitating intra-operative detection of Carbonic Anhydrase IX (CAIX)-expressing tumor lesions with chimeric monoclonal antibody (mAb) girentuximab, which has shown excellent imaging capabilities for ccRCC. Here we studied the potential of fluorescence imaging to detect ccRCC tumors with in nude mice with RCC xenografts by using mAb girentuximab conjugated with IRDye800CW, using SPECT imaging as a reference.

Methods

Groups of athymic BALB/c mice with subcutaneous CAIX-positive SK-RC-52 ccRCC tumors were injected intravenously with iodine-125 ($^{125}\text{I}$)-labeled girentuximab-IRDye800CW or $^{125}\text{I}$-labeled girentuximab. For determination of the specificity of the accumulation of the anti-CAIX antibody conjugate in ccRCC, separate groups of mice bearing a CAIX-positive (SK-RC-52) and CAIX-negative tumor (SK-RC-59) received $^{125}\text{I}$-girentuximab-IRDye800CW or $^{125}\text{I}$-labeled MOPC21-IRDye800CW (control mAb). Optical images and micro-SPECT images were acquired until 3 days after injection. Mice were euthanized after the last imaging session, and the biodistribution of the radiolabeled antibodies preparations was determined.

Results

Optical imaging and micro-SPECT imaging at 1 day after the injection of $^{125}\text{I}$-girentuximab-IRDye800CW showed clear delineation of the CAIX-expressing ccRCC xenografts, and image contrast improved with time. Fluorescence imaging
and biodistribution studies showed high and specific uptake of $^{125}$I-girentuximab-IRDye800CW in CAIX-positive ccrCC xenografts (SK-RC-52: 31.5 ± 9.6 %ID/g at 72 hours p.i.). Tumor uptake was specific, as very low uptake of $^{125}$I-girentuximab-IRDye800CW was noted in the CAIX-negative SK-RC-59 tumor (4.1 ± 1.5 %ID/g) and no uptake of the control mAb $^{125}$I-MOPC21-IRDye800CW was noted in the CAIX-positive SK-RC-52 tumor (1.2 ± 0.1 %ID/g).

**Conclusion**

Subcutaneous CAIX-expressing ccrCC xenografts were visualized by optical imaging with $^{125}$I-girentuximab-IRDye800CW. Optical images showed good concordance with micro-SPECT images. The accumulation of $^{125}$I-girentuximab-IRDye800CW in ccrCC tumors was high and specific. Girentuximab-IRDye800CW potentially could be used for the intraoperative detection CAIX-expressing tumors and assessment of residual tumor in the resection margins or metastatic lesions in patients with ccrCC.
Near infrared imaging in ccRCC

Introduction

Renal cell carcinoma (RCC) accounts for approximately 2% of all malignancies [1]. Surgical resection is usually the first choice for localized disease, but when disease has metastasized, the prognosis is bleak. Because of the increased use of conventional radiological modalities, such as computed tomography (CT) and ultrasound (US), RCCs are being found more frequently and at earlier stages of the disease [2]. The recommended treatment for a localized RCC depends on the tumor size. Nephron-sparing surgery is performed for smaller RCCs (especially those ≤ 4 cm in diameter) [3] because overall survival data of T1 to T3 tumors are comparable for partial and radical nephrectomy [4]. Negative surgical resection margins are crucial for the clinical outcome because they prevent additional surgical interventions [5]. Therefore, adequate characterization and resection of the renal tumor and metastatic lymph nodes is of utmost importance.

The use of near-infrared (NIR) imaging probes for improved tumor characterization and delineation during surgery is increasing, although clinical application has been limited. The sensitivity of NIR imaging might be extremely high, and because of the low tissue autofluorescence in this part of the light spectrum, the use of NIR imaging probes could yield optimal visualization of tissues of interest. Unfortunately, the limited penetration depth of the emitted light of these agents hampers whole body applications [6].

Fluorescent agent IRDye800CW, a typical example of a NIR dye, emits NIR 789 nm photons when excited at 774 nm. IRDye800CW can be stably coupled to targeting molecules, such as peptides and monoclonal antibodies (mAbs), and can be detected with a NIR imaging camera [7, 8]. For clear cell RCC (ccRCC) -the most common subtype of RCC, present in approximately 85% of the patients presenting with a malignant renal tumor- the chimeric mAb girentuximab (also known as cG250) could serve as a such a targeting molecule. Girentuximab specifically recognizes Carbonic Anhydrase
IX (CAIX), a tumor-associated antigen ubiquitously expressed in ccRCC. The use of girentuximab in radioimmunoscintigraphy and radioimmunotherapy to detect or treat ccRCC has been investigated extensively [9, 10]. Because of the high-level and specific targeting and accumulation of girentuximab in ccRCC tumors, we hypothesized that this antibody could also act as a carrier for the delivery of IRDye800CW in these tumors. The intravenous administration of girentuximab-IRDye800CW before (partial) nephrectomy might facilitate the sensitive, in vivo, intraoperative detection of positive resection margins, metastatic lymph nodes, or distant ccRCC metastases.

Here we characterized girentuximab-IRDye800CW in a subcutaneous mouse model. The antibody-IRDye800CW conjugate was radiolabeled to quantitatively determine the biodistribution. Studies were conducted to demonstrate tumor-specific and antigen-mediated uptake of the girentuximab-IRDye800CW conjugate. In addition, tumor targeting and biodistribution of the conjugate was compared with those of unconjugated radiolabeled girentuximab. This NIR imaging system could potentially be used intraoperatively to enhance tumor visualization and resection in the near future.

**Materials and methods**

**ccRCC tumors in nude mice**

**Cell lines**

The ccRCC cell lines SK-RC-52 (CAIX-positive) and SK-RC-59 (CAIX-negative) were derived from metastases of primary ccRCC patients as described by Ebert et al [11]. Cells were cultured and washed as described previously [12].
Animal model

The animal experiments were approved by the Internal Review Board of the Radboud University Medical Center, Nijmegen, The Netherlands, and were performed in accordance with that organization’s guidelines. Animals were housed and fed according to the Dutch animal welfare regulations. For obtaining subcutaneous tumors, 2x10^6-3x10^6 cells were injected subcutaneously into the flanks of 6- to 8-week-old male BALB/c nu/nu mice (Janvier) and tumors grew to a size of 50-200 mm^3 within 2-3 weeks.

Imaging agents and labeling

mAb girentuximab

The isolation and immunohistochemical reactivity of mAb G250 have been described elsewhere [13]. For reducing the immunogenicity of murine G250 in humans, a chimeric version (girentuximab) has been developed [14]. mAb girentuximab is reactive with the transmembrane glycoprotein CAIX (K_a = 4 x 10^9 M^-1). Expression of CAIX on the cell surface of ccRCC cells is ubiquitous (>95%), whereas expression on normal tissues is restricted to the epithelial structures of the upper gastrointestinal tract and larger bile ducts [15, 16].

mAb MOPC21

MOPC21, a murine IgG1 mAb (Sigma-Aldrich) that is not directed against any known antigen, was used as a nonspecific control mAb in these studies.
Conjugation, radiolabeling and quality control

Conjugation of mAb girentuximab and MOPC21 with IRDye800CW (LICOR biosciences) was performed according to the supplier’s protocol. One milligram of mAb was incubated with 0.03 mg IRDye800CW for 2 hours at room temperature in 1 mL of 10 mM phosphate buffer (pH 8.5). The reaction mixture was transferred into a Slide-A-Lyzer cassette (molecular weight cut-off: 10,000 Da) (Pierce) and dialyzed extensively against phosphate-buffered saline for 3 days to remove the unconjugated IRDye800CW. On average 1.6 IRDye800CW molecules were conjugated per mAb molecule, as determined spectrophotometrically. The concentration of the conjugate was adjusted to 1 mg/mL, and aliquots were stored in the dark at -20 °C until use. Girentuximab and girentuximab-IRDye800CW were radioiodinated with iodine-125 (\( ^{125}\text{I} \)) (PerkinElmer) by the IODO-GEN (Pierce) method [14]. \( ^{125}\text{I} \)-girentuximab was purified by gel filtration on a PD10 column (Amersham Biosciences); elution was done with phosphate-buffered saline containing 0.5% bovine serum albumin. The specific activities of \( ^{125}\text{I} \)-girentuximab and \( ^{125}\text{I} \)-girentuximab-IRDye800CW was 4.0 and 7.8 MBq/μg, respectively. In all experiments, a mAb dose of 5 μg was used.

The radiochemical purity of the radiolabeled mAb preparations was determined by instant thin-layer chromatography with silica gel strips (Biodex Medical Systems) and 0.1 M citrate buffer (pH 5.0) as the mobile phase. The labeling efficiency exceeded 90% for all antibody preparations. After purification by gel filtration on a PD10 column, the radiochemical purity of all preparations used in the studies exceeded 95%. The immunoreactive fractions of all radiolabeled girentuximab preparations, as determined on freshly trypsinized SK-RC-52 ccRCC cells essentially as described by Lindmo et al. [17], were greater than or equal to 79% for \( ^{125}\text{I} \)-girentuximab and greater than or equal to 67% for \( ^{125}\text{I} \)-girentuximab-IRDye800CW.
Optical imaging and microSPECT imaging

Images were recorded at 1 hour, 1 day, and 3 days after intravenous mAb injection. Optical images were acquired with an IVIS Lumina imaging system (recording time, 1-3 min; binning factor, 4; emission filter, ICG; field of view, 12.5; excitation filter, 745 nm) (Caliper life sciences). Mice were placed in the prone position in the scanner and body temperature was maintained at 37 °C with a heated imaging stage. All animals were gas-anesthetized with a mixture of oxygen, N₂O, and isoflurane. Micro-SPECT images were acquired with a U-SPECT II micro-SPECT scanner (MILabs) by the use of the GP-RM 1.0 mm collimator tube with 75 pinholes [18]. SPECT images were reconstructed with ordered-subset maximization expectation (6 iterations; 16 subsets; voxel size 0.1875 mm) using the U-SPECT-Rec software (MILabs).

Study design

Changes in girentuximab biodistribution as a result of the conjugation of the mAb to IRDye800CW were assessed with 2 groups of 5 mice. Mice with a subcutaneous SK-RC-52 tumor were injected with either ¹²⁵I-labeled girentuximab-IRDye800CW or unconjugated ¹²⁵I-labeled girentuximab. Both groups were studied with micro-SPECT imaging, and the group receiving ¹²⁵I-girentuximab-IRDye800CW was also studied with optical imaging. Biodistribution studies were performed for both groups.

For determination of whether tumor targeting of the girentuximab constructs was CAIX antigen-mediated, 2 groups of 6 mice with subcutaneous CAIX-positive tumor (SK-RC-52) in the left flank and a subcutaneous CAIX-negative tumor (SK-RC-59) in the right flank were used. One group of mice received ¹²⁵I-girentuximab-IRDye800CW, and the other group received nonspecific ¹²⁵I-labeled MOPC21-IRDye800CW. Both groups
were studied with optical imaging as well as micro-SPECT imaging, and biodistribution studies were performed.

The specificity of girentuximab targeting to CAIX-expressing ccRCC tumors was studied with an additional group of 5 SK-RC-52–bearing mice. These mice were coinjected with \(^{125}\text{I}\)-girentuximab-IRDye800CW and an excess (500 mg) of unlabeled girentuximab. Again, optical imaging as well as micro-SPECT imaging and biodistribution studies were performed.

For assessment of the feasibility of image-guided surgery for ccRCC tumors, 5 mice with subcutaneous CAIX-expressing tumors were injected intravenously with \(^{125}\text{I}\)-girentuximab-IRDye800CW. Tumors were visualized with fluorescence imaging at 3 days after injection. Afterwards, the tumors were resected, and imaging of the mice was performed again to evaluate whether radical tumor resection was achieved.

**Biodistribution studies**

After the last images were acquired (3 days after injection), mice were euthanized, and the biodistribution of the radiolabel was determined. Tumors and samples of normal tissues (blood, muscle, lung, spleen, kidney, liver, small intestine, and stomach) were dissected and weighed, and counts were obtained with a γ counter (1480 Wizard 399; PerkinElmer). The biodistribution results are reported as the percentage injected dose (%ID) per gram of tissue (%ID/g). The thyroid was also dissected, and counts were obtained; the results for this organ are expressed as the %ID. Counts for injection standards were obtained simultaneously to correct for radioactive decay.
Statistical analysis

Statistical analysis was performed using t tests. Differences were considered significant at \( p \) value of less than 0.05 (2-sided). All values are expressed as mean ± SD.

Results

Optical images of mice bearing a subcutaneous CAIX-positive SK-RC-52 tumor and injected with \(^{125}\)I-girentuximab-IRDye800CW showed targeting of the tumor by the IRDye800CW-conjugated antibody preparation at 1 and 3 days after injection (Figure 1B). No tumor uptake was detected at 1 hour after injection (data not shown). Micro-SPECT images of the same animals confirmed the accumulation of \(^{125}\)I-girentuximab-IRDye800CW in the CAIX-expressing tumor (Figure 1A). The biodistribution of the radiolabel in these mice is summarized in Figure 1D. The data (SK-RC-52 uptake, 6.9 ± 1.1 %ID/g; mean tumor weight, 0.09 ± 0.03 g) confirmed the preferential uptake of \(^{125}\)I-girentuximab-IRDye800CW in the tumor, as visualized on the optical images and the micro-SPECT images.

Micro-SPECT images of SK-RC-52 tumor–bearing mice injected with \(^{125}\)I-girentuximab showed tumor uptake at 1 and 3 days after injection (Figure 1C). No tumor uptake was detected at 1 hour after injection (data not shown). The biodistribution data for \(^{125}\)I-girentuximab confirmed the preferential tumor accumulation observed on the micro-SPECT images (14.8 ± 3.3 %ID/g; mean tumor weight, 0.09 ± 0.06 g) (Figure 1D).

At 3 days after injection, the levels of \(^{125}\)I-girentuximab-IRDye800CW in blood were significantly lower than those of \(^{125}\)I-girentuximab (2.7 ± 0.35 vs 7.1 ± 1.3 %ID/g, respectively; \( p = 0.001 \)), suggesting faster clearance of the IRDye800CW conjugated antibody preparation from the blood. However, tumor-to-blood ratios
Figure 1. (A) Micro-SPECT images of mouse bearing SK-RC-52 tumor on right flank (arrow) at 1 and 3 days after injection of $^{125}$I-girentuximab-IRDye800CW. In addition to tumor uptake, minimal uptake in heart and thyroid was observed. (B) Optical images of same mouse at 1 and 3 days after injection (same image settings were used for days 1 and 3). Some reflectance-induced image artifacts on backs of mice were observed. (C) Micro-SPECT images of mouse bearing SK-RC-52 tumor on right flank (arrow) at 1 and 3 days after injection of $^{125}$I-girentuximab. (D) Biodistribution of $^{125}$I-girentuximab-IRDye800CW and $^{125}$I-girentuximab in mice with subcutaneous SK-RC-52 tumor at 3 days after injection. Values are expressed as mean ± SD. T/B ratio = tumor-to-blood ratio.
did not differ significantly between the IRDye800CW-conjugated antibody and the unconjugated antibody (2.6 ± 0.34 vs 2.1 ± 0.34 %ID/g, respectively; \( p = 0.053 \)).

In the second experiment, we investigated the specificity of the CAIX-mediated targeting of girentuximab by administering \(^{125}\text{I}-\text{girentuximab-IRDye800CW}\) or \(^{125}\text{I}-\text{MOPC21-IRDye800CW}\) to mice with both a CAIX-positive tumor and a CAIX-negative tumor. Optical images and biodistribution data are shown in Figure 2. At 3 days after injection, the uptake of \(^{125}\text{I}-\text{girentuximab-IRDye800CW}\) in the CAIX-positive tumor was high (31.5 ± 9.6 %ID/g; tumor-to-blood ratio, 3.2 ± 2.0; mean tumor weight, 0.02 ± 0.01 g), whereas low uptake was observed in the CAIX-negative tumor (4.1 ± 1.5 %ID/g; tumor-to-blood ratio, 0.4 ± 0.2; mean tumor weight, 0.07 ± 0.11 g). Both tumor uptake and tumor-to-blood ratios were significantly different between CAIX-positive and CAIX-negative tumors (\( p = 0.003 \) and \( p = 0.018 \), respectively). No tumor uptake was detected at 1 hour after injection (data not shown).

Figure 2. (A) Optical images of mouse bearing CAIX-positive SK-RC-52 tumor on left flank and CAIX-negative SK-RC-59 tumor on right flank (arrows) at 1 and 3 days after injection of \(^{125}\text{I}-\text{girentuximab-IRDye800CW}\) (same image settings were used for days 1 and 3). Some reflectance-induced image artifacts on backs of mice were observed. (B) Biodistribution at 3 days after injection of \(^{125}\text{I}-\text{girentuximab-IRDye800CW}\) or \(^{125}\text{I}-\text{MOPC21-IRDye800CW}\) in mice with subcutaneous SK-RC-52 tumor and subcutaneous SK-RC-59 tumor. Values are expressed as mean ± SD.
Mice injected with the nonspecific control conjugate $^{125}$I-MOPC21-IRDye800CW showed significantly lower tumor uptake (for CAIX-positive SK-RC-52, 1.2 ± 0.1 %ID/g; tumor-to-blood ratio, 0.4 ± 0.1; mean tumor weight, 0.03 ± 0.01 g; for CAIX-negative SK-RC-59, 2.0 ± 0.5%ID/g; tumor-to-blood ratio, 0.56 ± 0.4; mean tumor weight, 0.01 ± 0.006 g) than mice injected with $^{125}$I-girentuximab-IRDye800CW ($p = 0.002$ and $p = 0.027$, respectively).

In the third experiment, the nonspecific uptake of girentuximab-IRDye800CW in the tumors was assessed by injecting a group of 3 mice bearing SK-RC-52 tumors with $^{125}$I-girentuximab-IRDye800CW and an excess of unlabeled girentuximab. Micro-SPECT images acquired at 1 hour, 1 day, and 3 days after injection did not show any uptake of the radiolabeled mAb in the CAIX-expressing tumors (data not shown). Biodistribution studies confirmed that the nonspecific uptake of the IRDye800CW-conjugated antibody in these tumors was very low (0.91 ± 0.25 %ID/g; tumor-to-blood ratio, 0.31 ± 0.06; mean tumor weight, 0.13 ± 0.08 g) (Figure 3).

In the fourth experiment, 5 mice with subcutaneous CAIX-expressing tumors received $^{125}$I-girentuximab-IRDye800CW intravenously, and tumors were visualized with fluorescence imaging at 3 days after injection. Subsequently, the tumors

![Figure 3. Ex vivo biodistribution of $^{125}$I-girentuximab-IRDye800CW and excess unlabeled girentuximab. T/B ratio = tumor-to-blood ratio.](image-url)
Near infrared imaging in ccRCC

were resected, and imaging of the mice was performed again to evaluate whether radical tumor resection was achieved. After resection, no residual tumor was detected by fluorescence imaging or by macroscopic examination (Figure 4).

Taken together, these results show that $^{125}$I-girentuximab-IRDye800CW can target CAIX-expressing tumors with high specificity and that this targeting can be assessed by micro-SPECT and NIR fluorescence imaging. Furthermore, these results demonstrate the feasibility of targeted fluorescence image-guided surgery for ccRCC tumors.

![Figure 4](image)

**Figure 4.** (Left) Preoperative fluorescence image of mouse with subcutaneous growing ccRCC tumor lesion at 3 days after injection with $^{125}$I-girentuximab-IRDye800CW. (Middle) Tumor lesion was subsequently removed by fluorescence image-guided surgery. (Right) After resection, no residual tumor was detected by fluorescence imaging or macroscopically.

**Discussion**

In the present study, girentuximab-IRDye800CW was characterized in a subcutaneous ccRCC xenograft model. Ex vivo biodistribution studies demonstrated high-level and specific targeting and high tumor-to-blood ratios for girentuximab-IRDye800CW in CAIX-expressing tumors. Compared with
girentuximab, the IRDye800CW-conjugated antibody preparation showed lower tumor uptake at 3 days after injection (Figure 1D), most likely because of faster clearance of the IRDye800CW-conjugated antibody from the circulation [8].

The preferential accumulation of dually labeled girentuximab in CAIX-expressing tumors was confirmed with both optical imaging and micro-SPECT imaging. Both imaging modalities showed that the radiolabeled girentuximab-IRDye800CW conjugate accumulated specifically in CAIX-positive tumors, whereas nonspecific mAb MOPC21-IRDye800CW conjugate did not. Moreover, girentuximab-IRDye800CW accumulated in CAIX-positive tumors but not in CAIX-negative tumors. These results clearly demonstrated that the accumulation of the girentuximab-IRDye800CW conjugate was antigen-mediated. The tracer uptake appeared to be higher in Figure 1B than in Figure 2A because of attenuation of the fluorescence signal in tissues.

The feasibility of using girentuximab-IRDye800CW in optical imaging and image-guided surgery for CAIX-expressing tumors suggests a potential role for this tracer in the intraoperative detection of positive surgical margins or tumor-infiltrated lymph nodes in patients with ccRCC tumors.

The results of the present study are in line with the results of other preclinical and clinical studies of dual-label imaging. Terwisscha van Scheltinga et al. [19] reported specific and sensitive detection of tumor lesions in vivo when they used fluorescence-labeled antibodies targeting vascular endothelial growth factor or human epidermal growth factor 2 in a breast cancer xenograft mouse model. The animals in that study received a second injection with the same antibodies labeled with a positron-emitting radionuclide, zirconium-89, to enable a comparison between fluorescence imaging and PET imaging. The addition of a radioactive tracer seems to be indispensable for intraoperative tumor detection because of the limited depth of tissue penetration.
of the optical signal. However, whether the coinjection of both an optical tracer and a radiotracer will produce the same results as the administration of a dually labeled tracer has not yet been determined. With coinjection, the biodistribution of the optical tracer could differ from that of the radiolabeled tracer; in contrast, with a dually labeled tracer, both signals originate from the same molecule. Nontargeted dually labeled tracers were applied for sentinel lymph node biopsy and resection after intratumoral injection of nanocolloid in a clinical setting [20]. The results clearly demonstrate improved lymph node detection, especially when positive nodes are located in lymph node-rich regions, for example in the case of prostate cancer or head and neck cancer [21, 22]. A study with patients who had ovarian cancer had already shown the feasibility and potential of this approach for intraoperative fluorescence imaging in a clinical setting [23]. Currently, a clinical trial is of the use of fluorescentce-labeled bevacizumab to enable image-guided surgery in patients with breast cancer is under way (NCT01508572). These preclinical and clinical studies demonstrate the feasibility of dual-modality imaging for image-guided surgery.

Although the present study demonstrated that the optical imaging of CAIX-expressing tumors is feasible, there are some limitations for the direct clinical translation of targeted fluorescence imaging in patients with ccRCC.

In the present study, we used $^{125}\text{I}$ for small-animal imaging and ex vivo biodistribution studies. Because of its long half life (59.4 days) and less favorable γ characteristics ($γ$ energy = 35 keV), $^{125}\text{I}$ is not suitable for SPECT imaging in the clinical setting. Labeling girentuximab-IRDye800CW with $^{124}\text{I}$ or with the residualizing radiometal indium-111 by use of the chelator DTPA could enable preoperative molecular imaging in patients with ccRCC tumors and thus enabling both pre- and perioperative imaging in ccRCC tumors [24-27]. Recently, Divgi et al. [26] assessed the accuracy of $^{124}\text{I}$-girentuximab PET/CT in a large cohort of patients with a primary renal
mass scheduled for surgery. The authors found high sensitivity and specificity of $^{124}$I-girentuximab PET/CT for detecting ccRCC lesions (86.2% and 85.9%, respectively); these results were concordant with prior experience with this tracer and suggest that this imaging modality could improve patient care in the preoperative setting [24].

A limitation of girentuximab-based imaging is that targeting depends on CAIX expression in tumor lesions. However, approximately 80% of malignant renal lesions are of the clear cell subtype, express CAIX, and therefore potentially can be imaged with girentuximab-IRDye800CW.

The results of the present study endorse the tumor-specific targeting of girentuximab and indicate that a new application for girentuximab-based imaging—namely, the intraoperative detection of ccRCC tumors, positive resection margins, and metastases with fluorescence imaging—may be feasible. This approach may lead to better cancer management. Further studies in which orthotopic or metastatic models are used to evaluate the sensitivity and potential added value of this approach are warranted.

**Conclusion**

CAIX-expressing subcutaneous ccRCC xenografts were visualized by optical imaging with $^{125}$I-girentuximab-IRDye800CW, with good concordance between fluorescence images and micro-SPECT images. The accumulation of $^{125}$I-girentuximab-IRDye800CW in ccRCC tumors was high and specific. The use of girentuximab-IRDye800CW for the intraoperative detection of CAIX-expressing tumor lesions and the assessment of residual tumor in resection margins or metastatic lesions in patients with ccRCC tumors may be feasible. Clinical studies are warranted to assess the safety, feasibility, and validity of this fluorescence imaging approach for patients with ccRCC tumors.
References

Chapter 4


Chapter 5

Radionuclide and fluorescence imaging of clear cell renal cell carcinoma using dual-labeled anti-Carbonic Anhydrase IX antibody G250

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Abstract

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. In this study, the feasibility of dual-modality imaging and image-guided surgery with a dual-labeled anti-Carbonic Anhydrase IX (CAIX) antibody preparation indium-111 ($^{111}$In)-DTPA-G250-IRDye800CW was evaluated in mice with intraperitoneally (i.p.) growing clear cell renal cell carcinoma (ccRCC) lesions.

Methods

BALB/c nu/nu mice with i.p. growing SK-RC-52 tumor lesions received 10 μg DTPA-G250-IRDye800CW labeled with 15 MBq of $^{111}$In (n=20) or 10 μg dual-labeled irrelevant control antibody NUH-82 (n=20). To evaluate when tumors could be detected, 4 mice per group were imaged weekly during five weeks with both SPECT/CT and the IVIS Lumina fluorescence imager, followed by ex vivo biodistribution studies.

Results

As early as one week after tumor cell inoculation, SPECT and fluorescence images showed clear delineation of the i.p. growing ccRCC lesions, with very good concordance between the SPECT/CT and fluorescence images. The high and specific accumulation of the dual-labeled antibody conjugate in the tumors was confirmed in the biodistribution studies. Maximum tumor uptake was observed one week after inoculation (G250: 58.5±18.7 vs NUH-82: 5.6 ± 2.3 %ID/g), but also on other time points high tumor uptake was observed.
Conclusions

This study demonstrates the feasibility of dual modality imaging with dual-labeled antibody $^{111}$In-DTPA-G250-IRDye800CW in a ccRCC model. Both SPECT and fluorescence images clearly showed specific accumulation of the tracer in the tumors, with excellent concordance between the imaging modalities. These results indicate that pre- and intraoperative detection of CAIX-expressing tumors, positive resection margins, and metastases might be feasible with this approach.
Introduction

To accomplish radical excision of tumor tissue in oncological surgery, intraoperative imaging may be applied. For example, for sentinel node procedures with the use of a gamma detector is a well-established technique in patients with melanoma, breast cancer, and other malignancies [1-3]. Recently, studies have shown that the success rate of these procedures increases with the addition of a near infrared (NIR) fluorescent label [4-6]. Although sentinel node procedures are helpful in guiding the surgeon to the first draining lymph node, they do not provide information about the nature of the lymph nodes. Tumor targeting molecules labeled with fluorescent dyes, however, can aid in both the intraoperative detection and accurate delineation of malignant lesions, and thus enabling image-guided surgery of primary tumors, metastases and the detection of positive resection margins [7].

A major drawback of all 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 [8-10].

The fluorophore IRDye800CW is a NIR dye that is manufactured under cGMP and approved for use in clinical trials. This fluorescent dye emits near infrared 789 nm photons when excited at 774 nm and can be coupled to tumor specific targeting molecules such as monoclonal antibodies (mAbs), and detected intraoperatively with a NIR imaging camera.

For clear cell RCC (ccRCC), which accounts for approximately 85% of the malignant renal tumors, G250 (chimeric mAb 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, both in the preclinical and clinical setting [11, 12]. G250 specifically recognizes Carbonic Anhydrase IX (CAIX), a tumor-associated antigen ubiquitously expressed in primary ccRCC and its metastases. Because of the high and specific targeting of G250 in all CAIX-positive lesions (both primary tumors and metastases), this antibody could act as a potent carrier for the delivery of both the radiotracer and the NIR dye in these lesions. As we recently described, such a dual modality imaging system enables preoperative Single Photon Emission Computed Tomography (SPECT) and intraoperative tumor detection by means of the radioactive and NIR signal [13]. In this study, we describe indium-111 ($^{111}$In)-DTPA-G250-IRDye800CW and show the feasibility of dual modality imaging and image-guided surgery in an intraperitoneal (i.p.) ccRCC model.

**Materials and methods**

**ccRCC tumors in nude mice**

*Animals*

All experiments were conducted in accordance with the principles laid out by the revised Dutch Act on Animal Experimentation (1997) and approved by the institutional Animal Welfare Committee of the Radboud University Nijmegen. Animals were housed and fed according to the Dutch animal welfare regulations. The experiments were performed in female nude BALB/c nu/nu mice (8-10 weeks old) weighing 20–25 g (Janvier, le Genest-Saint-Isle, France). Mice were accustomed to laboratory conditions for at least one week before experimental use and 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 induction of tumors

Intraperitoneal tumor growth was induced by i.p. injection of 0.2 mL of a suspension of \(3 \times 10^6\) SK-RC-52 cells, resulting in tumor nodules in the peritoneal cavity ranging from submillimeter to 5 mm in diameter after 1-5 weeks, which was in concordance with our previous experience with this model [14]. SK-RC-52 is a CAIX–expressing human ccRCC cell line which was acquired from the Memorial Sloan-Kettering Cancer Center (New York, USA). The cell line was previously described by Ebert et al. [15] and tested for authenticity by short tandem repeat profiling in August 2013 (DSMZ, Braunschweig, Germany).

Study design

Athymic female BALB/c nu/nu mice injected i.p. with \(3 \times 10^6\) SK-RC-52 cells as previously described [14]. After tumor cell inoculation, animals were divided in two groups of twenty mice. Based on our previous results in this i.p. xenograft model, an antibody protein dose of 10 μg was used [14]. One group was i.v. injected with 10 μg DTPA-G250-IRDye800CW labeled with 15 MBq \(^{111}\text{In}\) prior to imaging, the other group received 10 μg dual-labeled irrelevant isotype-matched control antibody NUH-82 [16]. To evaluate when tumors could be detected, 4 mice per group were imaged with both the microSPECT/CT and the IVIS Lumina optical imager 48 hours post injection (p.i.) weekly.
Imaging agents and labeling

Antibodies

G250 is a murine IgG1 mAb which is directed against the CAIX antigen expressed in ccRCC. NUH-82 is a murine IgG1 mAb not directed against any known antigen in the nude mice and was used as a negative control.

Conjugation, radiolabeling and fluorescent labeling

G250 and NUH-82 were conjugated with IRDye800CW-NHS (LICOR biosciences, Lincoln, NE, USA) according to the supplier’s protocol. Briefly, one mg mAb was incubated with a 6-fold excess 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 the conjugation with IRDye800CW, mAb G250 and NUH-82 were conjugated with diethylenetriaminepentaacetic acid (DTPA) as described previously [17]. The reaction mixture was transferred into a Slide-A-Lyzer cassette (molecular weight cut-off: 20,000 Da, Pierce, Rockford, IL, USA) and extensively dialysed against 0.25 M ammonium acetate (pH 5.4) for three days to remove the unconjugated IRDye800CW and DTPA. On average 1.1 IRDye800CW molecules were conjugated per mAb molecule as determined spectrophotometrically. The dual-labeled antibody preparations were stored in the dark at 4 °C until use.

$^{111}$InCl$_3$ was obtained from Covidien (Petten, The Netherlands). Ten μg G250 dissolved in 0.25 M ammonium acetate buffer (pH 5.4) and radiolabeled with 15 MBq $^{111}$InCl$_3$ in two volumes of 0.1 M MES (Sigma-Aldrich, Zwijndrecht, The Netherlands). After 20 minutes of incubation at room temperature, 50 mM EDTA was added to a final
Radionuclide and fluorescence imaging in ccRCC

concentration of 5 mM (Sigma-Aldrich, Zwijndrecht, The Netherlands). Radiochemical purity was determined by Instant Thin Layer Chromatography (ITLC) on silicagel, using 20 mM sodiumcitrate buffer (pH 5.0) as 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 multipinhole Ultra High Sensitive (UHS-M) collimator tube was used. Mice were anesthetized with a mixture of oxygen, N₂O and isoflurane and placed in a supine position in the scanner. Body temperature was maintained at 37 °C during the scan. Total scan time was approximately 60 minutes per animal for SPECT acquisition and 3 minutes for CT imaging. Images were reconstructed using the MILabs software (MILabs, Utrecht, the Netherlands). Immediately after completing of image acquisition, mice were euthanized, and placed in supine position in the optical scanner after removal of the abdominal skin. Optical images were acquired with the IVIS Lumina imaging system (recording time 1-3 min; binning factor medium; emission filter ICG; FOV C; excitation filter 745 nm) (Caliper life sciences, Hopkinton, MA, USA).

**Processing of the tumor specimens**

After dissection, tumor specimens were processed and 10-μm sections were cut. Ex vivo autoradiography studies were performed by exposing the sections to an imaging plate (Fuji Film BAS-SR 2025; Raytest, Straubenhardt, Germany) for 1 hour. Images were acquired with a radioluminography laser imager (Fuji Film BAS 1800 II system; Raytest) and analyzed with Aida Image Analyzer software (Raytest).
Sections were subsequently measured with the Odyssey 9120 fluorescence imaging system (800 nm channel, 42 μm resolution) (LICOR biosciences, Lincoln, NE, USA). CAIX expression was detected by staining with anti-CAIX mouse mAb M75, a hybridoma supernatant obtained from the HB-11128 ATCC cell line.

*Biodistribution studies*

After SPECT and optical image acquisitions, mice were euthanized and tissues dissected for *ex vivo* biodistribution studies. A tumor sample and samples of normal tissues (blood, muscle, heart, lung, spleen, pancreas, stomach, duodenum, kidney, liver) were dissected, weighed and counted in a γ-counter (1480 Wizard 3”, LKB/Wallace, Perkin-Elmer, Boston, MA, USA). Injection standards were also counted to correct for radioactive decay. Tissue uptake of the dual-labeled antibody was expressed as percentage of the injected dose per gram (%ID/g).

*Statistical methods*

Statistical analyses were performed using IBM SPSS Statistics version 20.0 (Chicago, IL, USA) and GraphPad Prism version 5.03 (San Diego, CA, USA). Differences in uptake of dual-labeled antibodies determined from the biodistribution studies were tested for significance using t-tests. Differences were considered significant at p<0.05, two-sided. All values are expressed as mean ± standard deviation (SD).
Results

SPECT/CT and optical images acquired 48 hours after injection of $^{111}$In-DTPA-G250-IRDye800CW clearly delineated the i.p. growing CAIX-expressing ccRCC lesions. As early as one week after tumor cell inoculation, tumor lesions were clearly visualized with both imaging modalities in 3 out of the 4 animals (Figure 1). In the animal where SPECT/CT and optical imaging did not indicate the presence of tumor lesions, no macroscopic tumor lesions were found during meticulous examination of the peritoneal cavity. Although deeper lying lesions (e.g. subhepatic or lesions within the mesentery) could not be detected with optical imaging due to the limited penetration depth of the emitted light, very good concordance between the SPECT/CT and optical images of more superficially located tumors was observed (Figure 1). All abdominal tumor lesions detected with optical imaging were subsequently removed. After removal, no residual tumor could be detected with optical imaging or macroscopically (Figure 2).

Some physiologic uptake of the conjugate in the liver was observed. In addition, retention of products of catabolism could be detected in the bladder. Excellent concordance between autoradiography and fluorescence imaging of the tumor sections was observed (Figure 3).

The high and specific accumulation of $^{111}$In-DTPA-G250-IRDye800CW in the CAIX-expressing tumors was confirmed by the biodistribution data (Figure 4). The highest tumor uptake of $^{111}$In-DTPA-G250-IRDye800CW (58.5 ± 18.7 %ID/g) was observed one week after tumor cell 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/g, $p =0.008$) one week after inoculation. In the course of the experiment, tumor and blood levels decreased and liver uptake increased (Figure 4). Tumor-to-liver ratios steadily decreased from 2.55 after one week to 0.36 5 weeks after tumor induction, resulting in tumor-to-liver ratios of 2.16, 1.95 and 1.13,
Figure 1. SPECT/CT and fluorescence images of mice one week after tumor cell inoculation. Clear visualization of an i.p. growing ccRCC tumor lesion was observed 48 hours p.i. of $^{111}$In-DTPA-G250-IRDye800CW (A). Some physiological uptake of the tracer in the liver and retention of catabolic products in the bladder and urine was observed. In the animal receiving dual-labeled NUH-82 (B), a parasplenic lesion was detected macroscopically (blue circle), but this lesion was not detected by either imaging modality.

Figure 2. Preoperative SPECT/CT image (left) and fluorescence image (middle) of a mouse with an i.p. growing ccRCC tumor lesion, 48 hours p.i. of the tumor targeting dual-labeled imaging probe $^{111}$In-DTPA-G250-IRDye800CW. The tracer also shows some physiological uptake in the liver. The abdominal tumor lesion (arrow) was subsequently removed by fluorescence image-guided surgery (right). After resection, no residual tumor was detected with optical imaging or macroscopically.
after 2, 3 and 4 weeks, respectively and these were significantly higher than at 5 weeks after inoculation ($p = 0.005$, $p = 0.013$ and $p = 0.004$, respectively).

Discussion

In this study dual modality imaging and image-guided surgery of tumor lesions with the dual-labeled tracer $^{111}$In-DTPA-G250-IRDye800CW was tested in a ccRCC model. Both SPECT and fluorescence images showed high and specific accumulation of the dual-labeled G250 in the CAIX expressing tumor lesions, with very good concordance between the two modalities (Figure 1). After the imaged-guided surgery, no residual tumor was detected with optical imaging or macroscopically (Figure 2) indicating that pre- 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 the CAIX-expressing tumor lesions was confirmed by the biodistribution studies (Figure 4). Accumulation of the tracer in the tumor lesions was highly dependent on the total tumor load, in concordance with previous studies [14, 18]. Especially in the mice with a high tumor load (five weeks after tumor cell inoculation), tumor uptake levels were significantly lower than tumor uptake levels in animals with lower tumor burden. In the course of the experiment, gradually decreasing tumor accumulation along with lower blood levels and increasing liver uptake was observed (Figure 4). In addition, tumor-to-liver ratios decreased dramatically (Figure 4). This could be the result of antibody conjugate binding to circulating antigen in the blood [19], which will lead to rapid clearance of the antibody-antigen immune complexes via the liver.

The results of our study indicate that intraoperative detection of primary ccRCC tumors and positive resection margins might be feasible in patients. The detection of positive resection margins could theoretically either take place intracorporally 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 over the years [20-27], indicating that clinical translation of this dual-labeled tracer is feasible. In addition, labeling the antibody preparation with $^{111}$In enables high quality pre-operative whole body SPECT imaging, which can be helpful in clinical decision making in these patients [24].

Although the results of the present study are promising, several parameters may affect the visualization of ccRCC lesions. Firstly, targeting of the antibody and thus the intensity of both the radioactive and the fluorescent signal is dependent
on the degree and extent of CAIX expression. Although CAIX expression in most ccRCCs is homogeneous, G250-uptake is quite heterogeneous due to differences in perfusion, vascular permeability, and interstitial fluid pressure [25, 28]. As a result, uptake of the G250-based tracer may vary substantially between lesions.
Secondly, the antibody/dye molar substitution ratio is known to heavily affect the \textit{in vivo} characteristics of the antibody conjugate, including biological properties, signal-to-background ratio, clearance and biodistribution [29, 30]. Indeed, at higher substitution ratios (≥ 3) rapid blood clearance and very high liver uptake was observed (data not shown).

Thirdly, the IVIS Lumina set-up differs from the currently available clinical surgical imaging systems. Although the i.p. ccRCC tumors were clearly visualized, we do not know whether the sensitivity of the preclinical IVIS Lumina system is comparable to the clinical systems. It is therefore unclear whether real-time imaging can be achieved in the operating theatre. A phase I clinical trial that will shed more light on the feasibility of pre- and intraoperative visualization of ccRCC lesions with $^{111}$In-girentuximab-IRDye800CW is planned.

**Conclusions**

In this study, the feasibility of dual-modality imaging with the dual-labeled antibody preparation $^{111}$In-DTPA-G250-IRDye800CW was evaluated in a model with i.p. growing ccRCC xenografts. Both SPECT and fluorescence images clearly showed specific accumulation of $^{111}$In-DTPA-G250-IRDye800CW in the ccRCC tumors, with very good concordance between the two imaging modalities. These results indicate that pre- and intraoperative detection of CAIX-expressing tumors, positive resection margins and metastases might be feasible with this dual-labeled antibody preparation. Clinical studies are warranted to assess safety, feasibility, and validity of this imaging approach in ccRCC patients.
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References


Optimizing Lutetium-177-anti-Carbonic Anhydrase IX radioimmunotherapy in an intraperitoneal clear cell renal cell carcinoma xenograft model

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Abstract

A new approach in the treatment of clear cell renal cell carcinoma (ccRCC) is radioimmunotherapy (RIT) using anti-Carbonic Anhydrase IX (CAIX) antibody G250. To investigate the potential of RIT with lutetium-177 (\(^{177}\text{Lu}\))-labeled G250, we conducted a protein dose escalation study and subsequently a RIT study with in mice with intraperitoneally growing ccRCC lesions. Mice with intraperitoneal xenografts were injected with 1, 3, 10, 30 or 100 μg G250 labeled with 10 MBq indium-111 (\(^{111}\text{In}\)) to determine the optimal protein dose. The optimal protein dose determined with imaging and biodistribution studies was used in a subsequent RIT experiment in three groups of 10 mice with intraperitoneal SK-RC-52 tumors. One group received 13 MBq \(^{177}\text{Lu}\)-DOTA-G250, a control group received 13 MBq nonspecific \(^{177}\text{Lu}\)-MOPC21, and the second control group was not treated and received 20 MBq \(^{111}\text{In}\)-DOTA-G250.

The optimal G250 protein dose to target ccRCC in this model was 10 μg G250. Treatment with 13 MBq \(^{177}\text{Lu}\)-DOTA-G250 was well tolerated and resulted in significantly prolonged median survival (139 days), compared to controls (49-53 days, \(p = 0.015\)), indicating that RIT has potential in this metastatic ccRCC model.
Introduction

The approval of targeted therapies such as tyrosine kinase inhibitors (TKIs) and inhibitors of mammalian target of rapamycin has revolutionized the treatment of advanced clear cell renal cell carcinoma (ccRCC) during the last decade. Sunitinib, pazopanib, temsirolimus, and bevacizumab in combination with interferon-α have improved progression-free survival and are now approved as first line treatments for metastatic ccRCC [1-5]. Unfortunately, these agents have several disadvantages. First, they are associated with sometimes severe side effects, such as diarrhea, hypertension, fatigue and hand-foot syndrome [6]. Moreover, treatment with these drugs is not curative, implicating chronic treatment. In addition, discontinuation of therapy may lead to flare-up of disease activity in some of the patients [7]. Continued treatment despite disease progression or a switch to another angiogenesis inhibitor with a relatively short interval is therefore necessary.

The need for a less toxic therapeutic option with good and durable responses led to studies targeting the Carbonic Anhydrase IX (CAIX) using monoclonal antibody (mAb) G250 (chimeric mAb also known as girentuximab). G250 specifically binds to CAIX, which is expressed in approximately 94% of the ccRCC and expression in normal tissues is limited [8]. This preferential high expression in ccRCC has been used in many preclinical and clinical studies for imaging and therapy of ccRCC [9].

Due to the limited efficacy observed in the first clinical trials using iodine-131 ($^{131}$I)-girentuximab, the search for more suitable radionuclides was initiated. A preclinical study by Brouwers and colleagues revealed superior therapeutic efficacy of lutetium-177 ($^{177}$Lu), yttrium-90, or rhenium-186 labeled girentuximab as compared to $^{131}$I-labeled girentuximab. As tumor growth in mice with subcutaneous (s.c.) was delayed most effectively by the $^{177}$Lu-labeled antibody [10], a phase I dose escalation trial with $^{177}$Lu-girentuximab in patients with advanced ccRCC was initiated. The results
of this trial were very promising, as $^{177}$Lu-girentuximab RIT was generally well tolerated and resulted in disease stabilization in the majority of patients [11]. However, now the question arises how to combine RIT with other treatment modalities such as tyrosine kinase inhibitors, as we recently found that the uptake of girentuximab is markedly reduced during treatment with these agents [12]. To design the optimal treatment strategy, additional preclinical studies regarding the sequencing of therapies are of utmost importance.

In preclinical studies to date, tumor growth was induced by subcutaneous injection of tumor cells or grafting of harvested xenograft tissue, resulting in a single, palpable, visible tumor [10, 13]. Although the growth of subcutaneous tumors can be monitored easily with caliper measurements, subcutaneously growing xenografts differ substantially from tumor lesions in patients, particularly with respect to blood supply and physiology [14, 15]. In this study, we evaluated the potential of combined radioimmunodetection and RIT with murine G250 in an intraperitoneal ccRCC xenograft model that may more closely mimic human metastatic ccRCC.

**Materials and methods**

**Antibodies**

G250 is a murine IgG1 mAb which is directed against the CAIX antigen expressed in ccRCC. The G250 antibody was affinity purified on a Protein-A column from the supernatant of G250 hybridoma cell cultures. MOPC21 (Sigma-Aldrich, Zwijndrecht, The Netherlands) is a murine IgG1 mAb not directed against any known antigen and was used as a negative control in the RIT experiment.
Conjugation, radiolabeling and quality control

For the antibody dose escalation study both G250 and MOPC21 were conjugated with isothiocyanato-benzyl-diethylenetriaminepentaacetic acid (ITC-DTPA) as described previously [10].

For biodistribution studies, DTPA-conjugated G250 was incubated with $^{111}$In (Covidien BV, Petten, The Netherlands) in 0.1 M MES buffer, pH 5.5, at room temperature, under strict metal-free conditions for 20 minutes as described previously [16]. The specific activity of the antibody preparation was 1.07 MBq/μg (yield 98%). The protein dose was adjusted to 3, 10, 30 or 100 μg by adding unlabeled G250. For single-photon emission computed tomography (SPECT)/computed tomography (CT) studies, 67.2 μg DTPA-conjugated G250 was incubated with 403 MBq $^{111}$In. This preparation was purified on a PD10 column. The specific activity of $^{111}$In-DTPA-G250 used for imaging studies was 3.4 MBq/μg (overall yield 19%). Again, the protein dose for the different groups was adjusted by adding unlabeled G250. Ten megabecquerels of $^{111}$In-DTPA-G250 was administered intravenously via the tail vein (0.2 mL).

For the therapy experiment isothiocyanato-benzyl-1,4,7,10-tetraazacyclododecane-11-tetraacetic acid (ITC-DOTA) was used as bifunctional chelator because of the slightly better stability with $^{177}$Lu [10]. DOTA conjugation was performed essentially as described by Lewis et al. [17]. No-carrier-added $^{177}$Lu was acquired from ITG (Gärching, Germany). For RIT, antibody-DOTA conjugates were radiolabeled with $^{177}$Lu (G250: 46% yield, MOPC21 53% yield) or with $^{111}$In (74% yield). The radiochemical purity of the radiolabeled antibody preparations in the RIT experiment all exceeded 95%. The specific activity of $^{177}$Lu-DOTA-G250, $^{177}$Lu-DOTA-MOPC21 and $^{111}$In-DOTA-G250 was 1.3, 1.3 and 1.6 MBq/μg, respectively.
Cell culture

Tumor growth was induced by an intraperitoneal injection of 0.2 mL of a suspension of $3 \times 10^6$ SK-RC-52 cells, a CAIX–expressing human ccRCC cell line [18], resulting in tumor nodules (submillimeter to 3 mm in diameter) in the peritoneal cavity after 2 to 4 weeks.

Animals

All experiments were conducted in accordance with the principles laid out by the revised Dutch Act on Animal Experimentation (1997) and approved by the institutional Animal Welfare Committee of the Radboud University Nijmegen. Animals were housed and fed according to the Dutch animal welfare regulations. The experiments were performed in female nude BALB/c nu/nu mice (8–10 weeks old) weighing 20 to 25 g (Janvier, le Genest-Saint-Isle, France). Mice were accustomed to laboratory conditions for at least 1 week before experimental use and housed under nonsterile standard conditions in filtertopped cages with free access to animal chow and water.

Protein dose escalation study

Twenty-five female athymic BALB/c nu/nu mice were injected with SK-RC-52 cells as described above and divided in five groups. Three weeks after inoculation, the mice were injected with 3, 10, 30 or 100 μg G250-DTPA labeled with 10 MBq $^{111}$In. At the 1 μg dose level, the protein dose was too low for labeling with activity doses required for SPECT imaging. Therefore, the conjugate in this group was labeled with 1 MBq only, which is sufficient to determine the biodistribution of the radiolabeled antibody.
Imaging with the U-SPECT II scanner (MILabs, Utrecht, The Netherlands) was performed in the 3, 10, 30, or 100 mg groups 48 hours postinjection of $^{111}$In-DTPA-G250. A 1.0 mm diameter pinhole collimator tube was used. Mice were anesthetized with a mixture of oxygen, $\text{N}_2\text{O}$, and isoflurane and placed in a supine position in the scanner. Body temperature was maintained at 38°C during the scan. The total scan time was approximately 60 minutes per animal for SPECT acquisition and 3 minutes for CT imaging. Images were reconstructed using the MILabs software. Immediately after completion of image acquisition, mice were euthanized, and the intraperitoneal cavity was inspected meticulously for the presence of intraperitoneal tumor depositions. Tumor nodules and tissues were dissected, and their concentration of the radiolabel (\%ID/g) was determined. If no tumor lesions were detected, animals were excluded from the ex vivo biodistribution studies. A tumor sample and samples of normal tissues (blood, muscle, heart, lung, spleen, pancreas, stomach, duodenum, kidney, liver) were dissected, weighed, and counted in a gamma counter (1480 Wizard 3, LKB/Wallace, Perkin-Elmer, Boston, MA). Injection standards were also counted to correct for radioactive decay. Tissue uptake of the radiolabeled antibody was expressed as a percentage of the injected dose per gram (\%ID/g).

**RIT experiment**

For the RIT experiment, thirty female BALB/c nu/nu mice were injected with SK-RC-52 cells as described and divided into three groups. Two weeks after tumor cell inoculation, mice were injected with 10 μg 13 MBq $^{177}$Lu-DOTA-G250 (n=10), 13 MBq $^{177}$Lu-DOTA-MOPC21 (control group, n=10), or 20 MBq $^{111}$In-DOTA-G250 (control group, n=10).

The weight of the mice was measured weekly, and mice were euthanized in case of excessive palpable tumor growth, substantial weight loss or other signs of significant disease progression, as determined by trained biotechnicians who were blinded for
the treatment given to the mice. As in the protein dose escalation study, the presence of intraperitoneal tumor depositions was scored macroscopically. Animals were excluded from survival analyses if no tumor lesions were detected after inspection of the abdomen. SPECT/CT imaging to monitor disease progression was performed with 3-week intervals as described above. Primary endpoints were overall survival and toxicity.

**Statistical analysis**

Statistical analyses were performed using IBM SPSS Statistics version 20.0 (IBM, Armonk, NY) and GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA). Differences in uptake of radiolabeled antibodies were tested for significance using the nonparametric Kruskal-Wallis test and were considered significant at $p < 0.05$, two-sided. Tumor-to-blood (T/B) ratios were calculated by averaging the T/B ratios of the individual animals (tumor uptake (%ID/g) divided by blood activity (%ID/g)). Values are expressed as mean ± standard deviation. Median survival differences were tested for significance using a Mantel-Cox test and were considered significant at $p < 0.05$.

**Results**

**Protein dose escalation study**

Mice did not show clinical signs of discomfort 3 weeks after inoculation of the SK-RC-52 tumor cells. At that time, the intraperitoneal tumor nodules were not palpable. On macroscopic inspection of the opened abdomen, multiple solid tumors were found, predominantly located at the subhepatic, -splenic, and -phrenic spaces and within the mesentery (Figure 1). The number of tumor lesions per mouse was typically more than 10, ranging in diameter from less than a millimeter to 3 millimeter.
Eighteen of 25 mice (72%) had macroscopically visible tumors at dissection. Mice without macroscopically visible tumor growth were excluded from analyses.

SPECT/CT imaging showed high mAb uptake in the abdomen of mice at all protein dose levels. No clear differences in image quality were observed between the different groups. After the animals were sacrificed, all imaged areas coincided with intraperitoneal tumors which ranged from 1 to 2 mm, demonstrating preferential mAb uptake in the tumors. The biodistribution of $^{111}$In-DTPA-G250 was determined 48 hours post injection (Figure 2). Tumor uptake of the radiolabeled antibody was very high at all protein dose levels, with the highest mean uptake observed in the 10 μg group (54.9 ± 3.5), but the difference between the groups was not significant.

The highest T/B ratio was found in the 1 μg group (14.7 ± 11.6), mainly due to lower blood values, but the ratio was not statistically significant different from that of the other groups ($p = 0.297$). Significantly higher liver uptake was observed in the 3 μg group (23.0 ± 9.5)

**Figure 1.** Intraperitoneal tumor depositions close to the pancreas and spleen 21 days after tumor cell inoculation. Note the extensive neovascularization surrounding the tumors (arrow).
compared to the 10, 30 and 100 μg dose level groups \( p = 0.036 \). At protein dose levels \( \geq 10 \) ug favourable T/B ratios \( (8.5 \pm 3.3, 5.1 \pm 1.8 \) and \( 4.4 \pm 1.1 \), respectively) and lower liver uptake was observed, especially in the 10 μg group \( (T/B \) ratio \( 8.5 \pm 3.3) \). Therefore, it was concluded that the optimal G250 dose to target ccRCC in this model was 10 μg.

**RIT study**

Twenty-nine of 30 mice (97%) developed visible tumors during the experiment as assessed by meticulous macroscopic inspection of the peritoneal cavity. All mice survived the treatment and apart from mild transient weight loss (<15%) associated with the 177Lu-labeled antibody preparations, treatment was generally well tolerated. Three mice
died during the imaging procedures (injections or anesthesia) and were excluded from the analyses. One of these animals did not have macroscopically visible tumor growth.

Survival curves of the three groups are shown in Figure 3. Mice reached the humane endpoint when signs of significant disease progression or clinical deterioration (mainly severe weight loss) occurred, as judged by blinded biotechnicians. Lesion size ranged from <1.0 millimeter to 12 mm. Response to treatment was also monitored with $^{111}$In-DTPA-G250 SPECT/CT. As shown in Figure 4, progression of tumor lesions could easily be monitored in vivo, and when animals were sacrificed a close correlation between SPECT images and macroscopic tumors was observed.

Treatment with $^{177}$Lu-DOTA-G250 resulted in significantly prolonged median survival of 139 days, which is significantly longer than the survival of the mice in both control groups. The median survival of the mice that received $^{177}$Lu-DOTA-MOPC21 was 49 days and...
Optimizing radioimmunotherapy in ccRCC

Figure 4. In vivo SPECT/CT imaging of mice with intraperitoneal ccRCC tumors. Rapid disease progression occurred in an animal in the $^{111}$In-DOTA-G250 group, which reached a humane endpoint after 5 weeks (A). Another animal was treated with $^{177}$Lu-DOTA-G250 immediately after baseline imaging (B). Note the presence and progression of a tumor in the left side of the abdomen after 3 and 6 weeks (arrows). The tumor was not detected with SPECT/CT after 9 weeks and was not found during dissection, and possibly regressed due to RIT.

53 days in the $^{111}$In-DOTA-G250 treated group (Mantel-Cox test, $p = 0.015$). The study was terminated after 150 days, being the preset endpoint for assessment of survival.
Discussion

In this study, the potential of radioimmunodetection and RIT with radiolabeled G250 in an intraperitoneal ccRCC xenograft model was evaluated. The intraperitoneal model with the SK-RC-52 cell line proved reliable and highly reproducible, as substantial tumor growth was established in the vast majority of the inoculated mice (approximately in 85%).

Several studies indicate that substantial differences in blood supply and physiology exist within different tumor models [14, 15]. In current study, we opted for intraperitoneally growing tumor nodules to closer mimic metastatic disease with smaller tumor depositions in comparison to subcutaneous xenografts. Drawbacks of intraperitoneal tumor growth are that the lesions are not palpable the first weeks after induction, and that straightforward visual monitoring of the tumor growth is not possible. This makes in vivo imaging techniques indispensable if longitudinal quantification of tumor burden is required.

In the protein dose escalation study to determine the optimal antibody dose for in vivo targeting of the intraperitoneal ccRCC xenografts, mice did not show clinical signs of discomfort 3 weeks after inoculation of the SK-RC-52 tumor cells, nor were the intraperitoneal growing tumors palpable. Despite the small size of the intraperitoneal tumors, small depositions of approximately 1 to 2 mm and larger were well-visualized with SPECT/CT 48 hours post-injection of $^{111}$In-DTPA-G250, emphasizing the specific and high accumulation of mAb G250. No clear differences in image quality between the different dose levels were observed. Due to the low protein and radiation dose administered, it is highly unlikely that multiple $^{111}$In-DTPA-G250 injections used for disease monitoring will have any effect on tumor progression.

The protein dose escalation experiment demonstrated that optimal T/B ratios and low liver uptake levels were reached at a protein dose of 10 μg. Very high liver and low blood
levels were observed in the animals with normal tumor load after 3 weeks in the 1 and 3 μg groups. The mAb G250 biodistribution in the animals without any tumor depositions in these low protein dose groups was similar to the 10, 30 and 100 μg groups (data not shown). This suggests that the antibody distribution at low protein doses is heavily influenced by the presence of tumor. An inverse correlation was observed between blood levels and liver uptake at the lower protein doses. This correlation could be the result of binding of $^{111}$In-DTPA-G250 to circulating antigen in the blood due to antigen shedding by the tumor [19], which will lead to rapid clearance of the antibody-antigen immune complex via the liver, although we have no formal evidence of circulating antigen.

An additional advantage of an antibody dose of 10 μg or more is that it is easier to label the antibody with higher doses of radioactivity, which are required for RIT. In the subsequent RIT study, we found a significantly prolonged median survival in the group treated with $^{177}$Lu-DOTA-G250. The difference in median survival in this study (139 days) and our previous results in a s.c. model (300 days) [10], is likely due to the location of the tumor depositions, as the i.p. tumors often result in obstruction of the gastrointestinal tract, which subsequently leads to rapid weight loss and clinical deterioration of the animals. In both models, $^{177}$Lu-DOTA-G250 clearly shows therapeutic potential.

Recently, several clinical studies report severely hampered antibody targeting when radiolabeled antibodies are administered during TKI treatment [12, 20]. To successfully combine TKI treatment and RIT in the future, additional preclinical studies are warranted to investigate the optimal sequence and timing of both treatment modalities. The intraperitoneal ccRCC xenograft model used in current study mimics metastatic ccRCC and is therefore suitable for future experiments, for instance, to determine for how long antibody targeting to the tumor is reduced after cessation of the TKI treatment. Better understanding of underlying mechanisms could be an important step in the development of successful RIT strategies for ccRCC.
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We certify that all conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject matter or materials discussed in the manuscript (e.g., employment/affiliation, grants or funding, consultancies, honoraria, stock ownership or options, expert testimony, royalties, or patents filed, received, or pending), are the following: P.F.A. Mulders, W.J.G. Oyen, O.C. Boerman and E. Oosterwijk serve(d) on an advisory board for Wilex AG, Munich, Germany.
References


Chapter 7

Tyrosine kinase inhibitor sorafenib decreases $^{111}$Indium-girentuximab uptake in clear cell renal cell carcinoma patients

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Abstract

Tyrosine kinase inhibitors (TKIs) have revolutionized the treatment of metastatic clear cell renal cell carcinoma (RCC). Although TKIs have demonstrated good clinical efficacy, the lack of complete responses, the chronic nature of the treatment, and the side effects are clear disadvantages. An interesting new approach in the treatment of clear cell RCC (ccRCC) is antibody-mediated therapy with the chimeric anti–Carbonic Anhydrase IX (CAIX) antibody girentuximab (cG250). As the results of several girentuximab trials become available, the question arises of whether TKI treatment can be combined with girentuximab-based therapy. In this study, we assessed the effect of the widely used TKI sorafenib on the tumor-targeting potential of indium-111 (\(^{111}\text{In}\))-labeled girentuximab.

Methods

\(^{111}\text{In}\)-girentuximab imaging was performed on 15 patients suspected of having a renal malignancy, with surgery being part of their treatment plan. Of these, 10 patients were treated in a neoadjuvant setting with sorafenib (400 mg orally twice daily). Five patients received treatment during 1 week, and 5 patients received treatment during 4 weeks. In both sorafenib-treated groups, baseline and posttreatment tumor targeting of \(^{111}\text{In}\)-girentuximab were compared. Surgery was performed 3 days after the last image acquisition. Five additional patients were included as a control group and had only a single \(^{111}\text{In}\)-girentuximab injection and scintigraphy without any treatment. Distribution of \(^{111}\text{In}\)-girentuximab was determined scintigraphically ex vivo in a 1-cm lamella of the resected tumorous kidney. Expression of CAIX and of the vascular marker CD31 was determined immunohistochemically on specimens of both tumor and normal kidney tissue.
Results

Treatment with sorafenib resulted in a marked decrease of $^{111}$In-girentuximab uptake in the tumor in ccRCC patients, especially in the group treated for 4 weeks (mean change in both sorafenib-treated groups, -38.4%; range, 19.1% to -79.4%). Immunohistochemical analysis showed markedly reduced CD31 expression and vessel density in the sorafenib-treated groups but no differences in CAIX expression between the sorafenib-treated groups and the nontreated patients.

Conclusion

Treatment with sorafenib resulted in a treatment duration–dependent significantly decreased uptake of $^{111}$In-girentumab in ccRCC lesions. These results indicate that the efficacy of antibody-mediated treatment or diagnosis modalities is hampered by TKI treatment.
Introduction

Tyrosine kinase inhibitors (TKIs) have revolutionized the treatment of renal cell carcinoma (RCC) during the last five years. Sunitinib, sorafenib and pazopanib are now standard care in patients with advanced disease [1-3]. Although excellent results for progression-free survival have been achieved, there are limitations to the use of TKIs. First, these agents have a cytostatic rather than cytotoxic effect, and thus complete responses are rarely seen. Second, severe side effects such as hypertension, nausea, hand–foot skin reactions, and diarrhea can occur, and sustained low-grade toxicity can lead to dose adjustments. Third, the long-term effects of these agents are largely unknown [4]. Moreover, TKI treatment is chronic, and cessation of treatment may lead to flare-up of the disease, possibly due to rapid neovascularisation [5]. A new alternative approach in the treatment of clear cell RCC (ccRCC) is antibody-mediated therapy using the chimeric anti-Carbonic Anhydrase IX (CAIX) antibody girentuximab (cG250). CAIX is an antigen ubiquitously expressed in ccRCC [6, 7], but expression in normal tissues is low or absent [8]. The high and specific expression in ccRCC makes CAIX an excellent target for antibody-mediated therapy. Girentuximab has been studied in radioimmunotherapy trials in patients with metastatic disease [9-15] and is currently studied in trials in an adjuvant setting (ARISER, or Adjuvant RENCAREX [Wilex AG] Immunotherapy Trial to Study Efficacy in Nonmetastasised Renal Cell Carcinoma) [16]. Because we recently published the promising results for the most recent therapy trial [15], the question arises of whether antibody-mediated treatment can be combined with TKI treatment. In this trial, we aimed to determine the effect of the widely used TKI sorafenib on tumor targeting of indium-111 ($^{111}$In)-labeled chimeric anti-CAIX monoclonal antibody girentuximab in pre-operative patients presenting with a renal mass suspected of being RCC. To assess the effect of the duration of the treatment, patients were either treated during 1 week or during 4 weeks.
Materials and methods

Study design

Fifteen patients with renal masses suspected of being RCC and for whom surgery was planned were included in this study. Before inclusion at baseline (day -14 to day 0), a physical examination (including vital signs and weight) was performed, biochemical laboratory parameters were assessed, and a resting electrocardiogram was made. The study as approved by the institutional review board (CMO Arnhem-Nijmegen). Written informed consent was obtained from every patient. This trial is registered on www.clinicaltrials.gov, number NCT00602862. Two groups of 5 patients were infused with $^{111}$In-labeled girentuximab (100 MBq; 5 mg), and whole-body and SPECT images were obtained 7 days later. Imaging was followed by treatment with sorafenib (Nexavar [Bayer], 400mg twice a day orally) for 1 week or 4 weeks (Figure 1). All sorafenib treated patients underwent a second injection with $^{111}$In-girentuximab and imaging 7 days after injection. The sorafenib treatment was discontinued on the day of the last scan in both groups. Five patients served as a control group and received a single infusion of $^{111}$In-girentuximab 10 days before nephrectomy and were scanned 7 days later but did not receive sorafenib treatment. The study design is summarized in Figure 1.

Study drugs

Sorafenib was administered orally in a dosage of 400 mg twice a day. Dose interruptions and reductions were allowed when there were adverse events of grade III or higher according to the Common Terminology Criteria for Adverse Events, version 3.0 (National Cancer Institute).
For scintigraphic imaging, diethylenetriaminepentaacetic acid (DTPA) was conjugated to girentuximab (cG250;Wilex AG) at pH 9.5. A 50-fold molar excess of isothiocyanato-benzyl-DTPA was used to obtain a molar substitution ratio of 0.5–2.0. After conjugation, the unconjugated DTPA was removed by extensive dialysis against a 0.25 M ammonium acetate buffer, pH 5.4. Aliquots of the girentuximab-DTPA solution were placed into sterile vials (5 mg in 1.0 mL) and stored at -20°C until use. This procedure has been previously described in more detail [14]. After inclusion of a patient, the girentuximab-DTPA conjugate was labeled with 100 MBq of $^{111}$In (Covidien). The volume of $^{111}$In-DTPA-girentuximab was adjusted to 10 ml with NaCl 0.9%. Radiochemical purity was determined by instant thin-layer chromatography. All preparations used in this study exceeded 95%.
Imaging and quantitative image analysis

On the basis of previous studies, whole-body scans were acquired 7 days after injection using a double-head $\gamma$ camera (E.cam; Siemens Inc.), equipped with parallel-hole medium-energy collimators (symmetric 15% window over 172 and 247 keV) and a scan speed of 4 cm/min [17]. The sorafenib-treated groups were injected and scanned twice; the same imaging procedures were used for both scans.

In the group that was treated with sorafenib for 1 week, $^{111}$In-girentuximab was administered at 11 days before the start of sorafenib treatment and on the day that treatment began. Treatment with sorafenib was started 4 days after the first $^{111}$In-girentuximab image was acquired. Each time, scintigraphic imaging was performed 7 days post injection of $^{111}$In-girentuximab (Figure 1).

In the group that received 4 weeks of sorafenib, $^{111}$In-girentuximab was administered at 7 days before the start of sorafenib treatment and after 21 days of treatment, each time followed by scintigraphy 7 days after injection. The sorafenib treatment was stopped in both groups on the day of the last scan, and 3 days later a (partial) tumor nephrectomy was performed (Figure 1).

In the control group, patients were intravenously infused with $^{111}$In-girentuximab 10 days before nephrectomy and scanned 7 days after injection of the radiolabeled antibody.

Whole body planar images were analyzed quantitatively, as described by Visser et al [18]. Regions of interest were drawn around tumors and normal kidney. Targeting was expressed as the percentage injected dose per tissue weight (\%ID/g), assuming a tissue density of 1.0 g/ml. Tumor volumes were determined using Inveon Research Workplace software (Siemens Inc).
**Processing of the surgical specimen**

After nephrectomy, a 1-cm-thick slice of the resected tissue containing both tumor and normal tissue was scanned on the γ camera, using only a single detector. After imaging, the slice was cut into 1-cm³ blocks. Tissue blocks were weighed, and radioactivity in each sample was determined in a well-type γ counter (1480 Wizard; LKB/Wallace, Perkin-Elmer). After quantitative analysis, the tissue blocks were processed for immunohistochemical analysis.

**Immunohistochemical analysis**

CAIX expression was detected by staining with anti-CAIX mouse mAb M75, a hybridoma supernatant obtained from the HB-11128 ATCC cell line. CD31 staining was performed with murine anti-CD31 mAb JC70A (Dako). After staining, expression of CAIX and CD31 was scored by 4 independent observers on a scale ranging from undetectable (−) to low (±), moderate (+), high (++) and very high (+++).

**Statistics**

A nonparametric Wilcoxon signed-ranked test was performed to assess the change in $^{111}$In-girentuximab uptake in the tumor before and after sorafenib treatment. A $p$ value of less than 0.05 was considered significant.
Results

Patients

Patient characteristics are summarized in Table 1. Ten patients were treated with sorafenib (7 women and 3 men; median age, 62.4 y; range, 50.2–74.4 y). The tumors of 9 of these patients were ccRCC as determined by pathology (patients 2, 3, 4, 5, 7, and 9) or contained a ccRCC component (patients 1, 6, and 8). Patient 1 had a poorly differentiated tumor, partly ccRCC and partly rhabdoid RCC; patient 6 a partly ccRCC and partly papillary dedifferentiated tumor; and patient 8 a partly ccRCC with sarcomatoid dedifferentiated tumor. Patient 10 did not have ccRCC but a tumor of the chromophobe subtype.

In the control group (3 women and 2 men; median age, 62.2 y; range, 57.1–69.7 y), histopathologic examination revealed RCC in all patients. Of these patients, patient 11, 13, and 14 had the ccRCC subtype. The surgical specimen of patient 12 showed ccRCC, but a part was rhabdoid-dedifferentiated. The specimen of patient 15 revealed a type 2 papillary RCC (Table 1).

Treatment with sorafenib was generally well tolerated. Reported side effects were grade 3 skin toxicity according to the Common Terminology Criteria for Adverse Events (version 3; National Cancer Institute) in 2 patients, grade 2 diarrhea in 1 patient, and grade 2 stomatitis in 2 patients. No dose reductions or interruptions were necessary.
The effect of tyrosine kinase inhibitors on girentuximab uptake in ccRCC

Scintigraphy

In all patients with later-proven ccRCC, the renal tumors before neoadjuvant treatment were readily visualized with $^{111}$In-girentuximab. The results of the quantitative analysis are summarized in Table 1. Imaging of the 1-cm-thick slices of the resected tumorous tissue, followed by histologic analysis, revealed that $^{111}$In-girentuximab accumulated in areas of vital tumor tissue whereas normal kidney and necrotic or non–ccRCC parts showed much lower uptake (Figure 2). In one patient, the lung metastases as observed on CT were also visualized with scintigraphy (patient 8, Figure 3). In this patient, the uptake in the largest metastasis decreased by 74.8% after 4 weeks of sorafenib treatment. Patients 7 and 9 were diagnosed with bilateral renal masses and had girentuximab uptake in both lesions before treatment (patient 9, shown in Figure 4). Histologic analysis of the surgical specimens revealed that all these lesions were of the clear cell type (Table 1).

The mean $^{111}$In-girentuximab uptake in ccRCC renal tumors before sorafenib treatment was 0.013 %ID/g (range, 0.002–0.025 %ID/g). After 1 week of sorafenib treatment, the mean uptake decreased to 0.008 %ID/g (range, 0.002–0.02 %ID/g), indicating a mean change of -14.4% (range, 19.1% to -47.1%, $p = 0.225$). After 4 weeks of sorafenib treatment, the mean uptake decreased to 0.004 %ID/g (range, 0.002–0.01 %ID/g), which is equivalent to a mean change of -58.3% (range, -39.9% to -79.4%, $p = 0.028$) based on 6 lesions in 4 patients.

The tumors of all 5 patients who served as controls showed accumulation of $^{111}$In-girentuximab, with a mean uptake of 0.018 %ID/g. Uptake of $^{111}$In-girentuximab was also found in the papillary RCC in patient 15, albeit relatively low compared with the mean baseline uptake in the ccRCC tumors (0.003 %ID/g vs a mean of 0.018 %ID/g).
Figure 2. Macroscopic view (A) and scintigraphy (B) of 1 cm surgical lamella of patient 5. This patient presented with a largely necrotic renal mass with extension in the caval vein. Low uptake corresponds with necrotic parts, whereas the highest CAIX expression appears to be located in the caval vein extension.

Figure 3. Anterior planar images of patient 8 with a renal mass and lung metastases at baseline (A) and after 4 weeks of sorafenib treatment (B). Clear targeting is seen of $^{111}$In-girentuximab in the renal mass on the left side and lung metastases at baseline (arrows). Decreased targeting is observed after 4 weeks of sorafenib treatment.
The effect of tyrosine kinase inhibitors on girentuximab uptake in ccRCC

Immunohistochemical analysis

The results of the immunohistochemical analyses of the viable parts of the tumors are presented in Table 1. All ccRCC tumor specimens expressed CAIX (Table 1). Very low CAIX expression was also found in a vital tumor region of the surgical specimen of the papillary subtype from patient 15 (not shown).

Representative examples of post nephrectomy tumor specimens of each of the 3 groups are shown in Figure 5. From each tumor, multiple tumor blocks with the highest $^{111}$In uptake per gram of tissue (as determined in a $\gamma$ counter) were used.

High expression of CD31 was found in all the tumors in the control group. As expected, sorafenib treatment induced a marked reduction of vessel density, as observed

Figure 4. Posterior planar images of patient 9 with bilateral renal mass at baseline (A) and after 4 weeks sorafenib treatment (B). Clear targeting of $^{111}$In-girentuximab is seen in both lesions at baseline (arrows). Decreased targeting is observed after 4 weeks of sorafenib treatment.
Table 1: Patient Characteristics

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<th>Pt</th>
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<th>Age (y)</th>
<th>Sex</th>
<th>Type</th>
<th>Diameter (cm)</th>
<th>Volume (cm³)</th>
<th>Before</th>
<th>After</th>
<th>Δ%</th>
<th>Metastatic</th>
<th>Necrosis</th>
<th>M75</th>
<th>CD31</th>
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<td>59.4</td>
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<td>0.005</td>
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<td>0.005</td>
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<td>Yes</td>
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NA = not applicable
The effect of tyrosine kinase inhibitors on girentuximab uptake in ccRCC by CD31 immunohistochemistry. This effect was more pronounced in the group treated for 4 weeks than in the group that was treated for 1 week, suggesting that the decrease in vessel density was related to the duration of the sorafenib treatment.

Figure 5. Immunohistochemical analyses of vital tumor specimens from different groups. Neoadjuvant treatment with sorafenib resulted in enhanced necrosis (hematoxylin and eosin) and decreased vessel density (CD31) but did not have any effect on CAIX expression. Original magnification 20x.

Discussion

This study confirms previous studies showing that ccRCC lesions can be visualized with $^{111}$In-girentuximab imaging [17, 19]. High and specific uptake was seen in tumor lesions in all patients with ccRCC. Most importantly, we found that accumulation of girentuximab in ccRCC lesions is markedly reduced after sorafenib treatment in most patients.
The effect of sorafenib on the girentuximab uptake was more pronounced in patients that were treated with sorafenib for 4 weeks than in the group treated for 1 week (-58.3% vs -14.4%). All ccRCC tumor specimens in the current study were positive for CAIX, and we found no decrease in CAIX expression after the TKI treatment, indicating that sorafenib treatment did not down-regulate CAIX expression. We did observe decreased vessel densities in the sorafenib-treated groups when compared with the untreated group, making it highly likely that the reduced vessel density in the treated tumors results in poor perfusion and decreased uptake of $^{111}$In-girentuximab. The decrease in vessel density was most prominent after 4 weeks of treatment (Table 1; Figure 5). These results are in concordance with previous work of our group showing a similar effect of sorafenib on bevacizumab uptake [20] and with preclinical studies with other antibodies [21, 22]. Alternatively, the reduced $^{111}$In-girentuximab uptake could also be due to vascular normalization after sorafenib treatment [23]. Vascular normalization may have led to decreased vessel permeability, which could also have led to reduced accumulation of the antibody in the tumor. We also found CAIX expression in a patient presenting with a papillary RCC subtype (patient 15). Although CAIX expression has been reported in papillary RCC subtypes, the expression is much lower than in ccRCC, in terms of both incidence and level of expression [8, 24]. Strikingly, the CAIX expression in this particular patient was found in a vital tumor region within a necrotic part of the surgical specimen, suggesting this particular expression pattern is hypoxia driven [25].

The TKI-induced decrease of antibody uptake in the ccRCC lesions has major implications for future therapeutic regimens combining both TKIs and antibody-based treatments (either cold or radiolabeled). The results of the present study indicate that antibody-mediated treatment should preferably be given before TKI treatment. Alternatively, the antibody could be administered after TKI treatment has been stopped. The interval required between TKI cessation and antibody treatment still needs to be determined in future studies. In addition, the diagnostic
The effect of tyrosine kinase inhibitors on girentuximab uptake in ccRCC

performance of radiolabeled antibodies (immunoPET or immunoSPECT) may similarly be affected by TKI treatment. Currently, a clinical trial is ongoing that focuses on the potential of $^{124}$I-girentuximab immunoPET to detect early treatment response to sunitinib [26]. It will be interesting to see whether this study also indicates that TKIs interfere with the uptake of girentuximab and whether it is possible to evaluate the response to TKI treatment with girentuximab-based imaging.

There are some limitations to our study. First, there was a 3-day interval between the discontinuation of the sorafenib treatment and the (partial) nephrectomy. Therefore, the immunohistochemical findings may not fully reflect the actual effect caused by sorafenib, though significant histologic changes in this short interval are unlikely. Second, no biopsies of the renal lesions were taken at baseline, and therefore no intrapatient comparison of pre- and posttreatment tissue samples could be performed. In addition, vascular density may vary largely within and between RCC tumors. However, the CD31 expression in the samples from all patients treated with sorafenib for 4 weeks was lower than in the other samples, suggesting that the lower vessel density is a direct effect of the treatment. Although the changes in CD31 expression after sorafenib treatment were evident (Figure 5), microvessel densities in the treated groups could not be reliably quantified. In desmoplastic areas of treated tumors, we often observed cytoplasmic CD31 expression in nonendothelial cells. This phenomenon has been documented before and has been linked to the phenomenon of vasculogenic mimicry [27, 28]. Third, the current trial was not designed to assess the duration of the sorafenib induced decreased antibody uptake. It remains to be investigated for how long antibody targeting to the tumor remains at lower levels after cessation of sorafenib treatment. Data on this subject are limited. There are animal data suggesting increased antibody uptake after discontinuation of sunitinib treatment [21, 22], presumably due to rapid rebound vascularization. To date, no human data are available yet. Enhanced uptake of girentuximab shortly after discontinuation of TKI treatment
is an interesting feature for further investigation because it could be an important step in the development of successful radioimmunotherapy strategies for ccRCC.

**Conclusion**

This study confirms that ccRCC lesions can be identified with $^{111}$In-girentuximab scintigraphy. Moreover, we demonstrated that treatment with sorafenib markedly reduces the targeting of $^{111}$In-girentuximab in ccRCC lesions, suggesting that the effect of antibody-mediated treatment modalities would be profoundly hampered when directly combined with TKI treatment. Further research to evaluate the duration of this effect after discontinuation of TKI treatment is needed.

**Acknowledgments**

We thank Maichel van Riel and Miranda van de Veerdonk for their help with the labeling of the antibody. Sorafenib was provided by Bayer. Girentuximab was provided by Wilex.
The effect of tyrosine kinase inhibitors on girentuximab uptake in ccRCC

References


Chapter 8

Phase II study of Lutetium-177-labeled anti-Carbonic Anhydrase IX monoclonal antibody girentuximab in patients with advanced renal cell carcinoma: an interim report

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In preparation
Abstract

Introduction

Despite recent advances in treatment of metastatic clear cell renal cell carcinoma (ccRCC), i.e. the development of several angiogenesis inhibitors and mTOR inhibitors, the search for alternative treatment modalities that can induce durable responses with milder toxicity profiles continues. A phase II radioimmunotherapy (RIT) trial with lutetium-177 ($^{177}$Lu)-girentuximab was initiated based on the encouraging results of a phase I to evaluate the efficacy of this RIT approach in patients with advanced ccRCC. Results of the first 8 patients treated are reported.

Patients and methods

In this uncontrolled, non-randomized phase II trial, patients with progressive metastatic ccRCC who met the inclusion criteria received RIT with $^{177}$Lu labeled girentuximab if targeting of the antibody was observed after a diagnostic injection with indium-111 labeled girentuximab. Patients were eligible for another treatment cycle if they had at least stable disease (SD) on evaluation after 3 months according to RECIST v.1.1 and no prolonged grade 4 hematological toxicity was observed. Retreatment was at 75% of the previous activity dose with a maximum of 3 treatment cycles.

Results

Between August 2011 and August 2013, 8 patients enrolled in the study and received at least one infusion with $^{177}$Lu-girentuximab. SD after the first RIT was observed in 5 patients (62.5%), whereas progressive disease (PD) was seen in the other 3 patients. Of the 5 patients with SD, 2 patients were not eligible for retreatment due to prolonged
hematological toxicity after the first RIT cycle. After the second treatment cycle, continued SD was observed in 2 out of 3 patients. The treatment was generally well tolerated, but resulted in transient grade 3-4 hematological toxicity in all patients.

**Conclusions**

RIT with $^{177}$Lu-girentuximab resulted in disease stabilization in 5 out of 8 patients with progressive ccRCC. Apart from transient myelotoxicity, RIT was well tolerated.
**Introduction**

Despite advances in the treatment of metastatic renal cell carcinoma (RCC) with antibodies targeting the vascular endothelial growth factor (VEGF), tyrosine kinase inhibitors (TKIs) and mammalian target of rapamycin (mTOR) inhibitors in the past decade [1-10], there is still an unmet need for improved treatment options of this disease. The search for novel systemic treatment strategies with less toxicity and significant anti-tumor effect has led to therapeutic regimes using radiolabeled antibodies specifically targeting tumor-associated antigens expressed on tumor cells. For clear cell RCC (ccRCC), which accounts for approximately 85 percent of the malignant renal tumors, the radiolabeled chimeric monoclonal antibody girentuximab, is extensively investigated for both radioimmunodetection and radioimmunotherapy (RIT) [11-20]. Girentuximab specifically targets carbonic anhydrase IX (CAIX), a tumor-associated antigen ubiquitously expressed in primary ccRCC and its metastases but not found in the normal kidney [21-23]. Because of the specific targeting of girentuximab to CAIX-expressing lesions, this antibody is a potent carrier for tumor targeted delivery of β-emitting radionuclides [24]. Two independent phase I radioimmunotherapy (RIT) trials have been carried out using yttrium-90 (⁹⁰Y) or lutetium-177 (¹⁷⁷Lu) labeled girentuximab in patients with metastatic ccRCC [17, 25]. These trials were designed to assess the maximum tolerated dose (MTD), dosimetry, pharmacokinetics, and incidence of human anti-chimeric antibody (HACA) formation. While the results of the ⁹⁰Y-girentuximab trial are not yet available, ¹⁷⁷Lu-girentuximab RIT proved to be safe and well tolerable at activity dose levels as high as 2405 MBq/m². Moreover, 17 of 23 (74%) patients with advanced ccRCC demonstrated stable disease 3 months after the first infusion with ¹⁷⁷Lu-girentuximab [17]. Because of these encouraging results, a phase II trial at MTD was initiated. Here we report the interim results of this ongoing RIT trial in patients with advanced ccRCC.
Patients and methods

Adult patients with histopathologically proven metastatic ccRCC with evidence of progressive (>15% growth) or new RECIST v.1.1 evaluable lesions, with a maximum diameter of 5 centimeters, were eligible for participation. Additional inclusion criteria were a Karnofsky performance score > 70, white blood cell (WBC) count > 3.5 x 10⁹/l, thrombocyte count >150 x 10⁹/l, hemoglobin > 6 mmol/l, total bilirubin ≤ 2 x upper limit of normal (ULN), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) ≤ 3 x ULN (≤5 x ULN if liver metastases present), (MDRD > 40 ml/min and a negative pregnancy test for women of child bearing potential.

Previous treatment with external beam radiation, immunotherapy, VEGF inhibitors or mTOR inhibitors was allowed, when more than 4 weeks prior to study. Additional exclusion criteria included untreated hypercalcemia, cardiac disease with the New York Heart Association classification of III or IV and a life expectancy shorter than 4 months. In addition, patients with a history or clinical evidence of central nervous system (CNS) metastases were excluded, unless previously treated and asymptomatic, had no evidence of active CNS metastases for ≥ 3 months prior to enrollment, and had no requirement for steroids or enzyme inducing anticonvulsants in the last 14 days. Inclusion and exclusion criteria are listed in more detail in Table 1.

The study was registered on the clinicaltrials.gov website (NCT02002312) and was approved by the regional Medical Review Ethics Committee of the Region Arnhem-Nijmegen. All subjects provided written informed consent.
Table 1. Inclusion and exclusion criteria

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
<th>Exclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Patients with proven advanced and progressive RCC of the clear cell type</td>
<td>- Known or suspected CNS metastases including leptomeningeal metastases. History or clinical evidence of CNS metastases (unless they are previously-treated CNS metastases and patients meet all 3 of the following criteria: are asymptomatic, have had no evidence of active CNS metastases for ≥3 months prior to enrollment, and have had no requirement for steroids or enzyme inducing anticonvulsants in the last 14 days)</td>
</tr>
<tr>
<td>- Presence of evaluable lesions all &lt; 5 cm, according to RECIST v.1.1</td>
<td>- Untreated hypercalcemia</td>
</tr>
<tr>
<td>- Karnofsky Performance status: &gt; 70</td>
<td>- Chemotherapy, external beam radiation, immunotherapy or angiogenesis inhibitors or mTOR inhibitors within 4 weeks prior to study. Limited field external beam radiotherapy to prevent pathological fractures is allowed, when unirradiated, evaluable lesions elsewhere are present</td>
</tr>
<tr>
<td>- Laboratory values:</td>
<td>- Cardiac disease with New York Heart Association classification of III or IV</td>
</tr>
<tr>
<td>- White blood cells &gt; 3.5 x 10^9/l</td>
<td>- Patients who are pregnant, nursing or of reproductive potential and are not practicing an effective method of contraception</td>
</tr>
<tr>
<td>- Platelet count &gt; 150 x 10^9/l</td>
<td>- Any unrelated illness, e.g. active infection, inflammation, medical condition or laboratory abnormalities, which in the judgment of the investigator will significantly affect patients’ clinical status</td>
</tr>
<tr>
<td>- Hemoglobin &gt; 6 mmol/l</td>
<td>- Life expectancy shorter than 4 months</td>
</tr>
<tr>
<td>- Total bilirubin &lt; 2 x ULN</td>
<td></td>
</tr>
<tr>
<td>- ASAT, ALAT &lt; 3 x ULN (&lt; 5 x ULN if liver metastases present)</td>
<td></td>
</tr>
<tr>
<td>- MDRD ≥ 40 ml/min</td>
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</tr>
<tr>
<td>- Negative pregnancy test for women of child-bearing potential (urine or serum)</td>
<td></td>
</tr>
<tr>
<td>- Age over 18 years</td>
<td></td>
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<tr>
<td>- Written informed consent</td>
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</tbody>
</table>

RCC = renal cell carcinoma; RECIST = Response Evaluation Criteria in Solid Tumors; ULN = upper limit of normal; ASAT = aspartate aminotransferase; ALAT = alanine aminotransferase; MDRD = modification of diet in renal disease, formula to estimate Glomerular Filtration Rate; CNS = central nervous system; mTOR = mammalian target of rapamycin.
Study design

The primary endpoint of this study was to determine the therapeutic efficacy of $^{177}$Lu-girentuximab in patients with progressive metastatic ccRCC patients using the Response Evaluation Criteria in Solid Tumors (RECIST) v.1.1. Secondary objectives of this study were to assess the progression-free survival (PFS) and overall survival (OS) and to explore the toxicity of the $^{177}$Lu-girentuximab infusions. PFS was defined as the time measured from the day of first administration of $^{177}$Lu-girentuximab to first progression (either radiological or clinical) or death, whichever occurred first. OS was defined as the time from the first injection with $^{177}$Lu-girentuximab to the date of death from any cause.

A Simon two-stage minimax design was used to calculate the number of patients needed to include. The desirable response rate was set at 0.65, with $\alpha = 0.05$ and $\beta = 0.10$. Response was defined as at least stable disease (SD) on evaluation after 3 months according to RECIST v.1.1. Initially 6 patients were included to evaluate the therapeutic efficacy. After interim analysis for efficacy, 8 additional patients will be included in this study if a response rate of >0.25 is observed after the first treatment cycle in the first 6 patients.

Study procedures

Preparation of the radiolabeled antibody was performed as described earlier [17]. In brief, girentuximab was conjugated with 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA, Macrocyclics, Dallas, TX, USA) and labeled with either 185 MBq indium-111 ($^{111}$In) (Mallinckrodt Medical, Petten, The Netherlands) for the diagnostic infusion, or with 2405 MBq/m2 of $^{177}$Lu (ITG, Gärching, Germany) for the therapeutic infusion. Radiochemical purity exceeded 95% in all cases as confirmed by instant thin layer chromatography.
After screening, all eligible patients received an infusion with 185 MBq/10 mg $^{111}\text{In}$-DOTA-girentuximab to enable diagnostic imaging. Whole body planar and Single-Photon Emission Computed Tomography (SPECT/CT) images were obtained 2 hours, 2-4 and 5-7 days after injection to determine targeting of the radiolabeled monoclonal antibody. If at least one evaluable metastatic lesion was visualized with $^{111}\text{In}$-girentuximab imaging, $^{177}\text{Lu}$-DOTA-girentuximab (2405 MBq/m$^2$, 10 mg) was administered 9-10 days after infusion of $^{111}\text{In}$-DOTA-girentuximab. Patients were eligible for a maximum of three treatment cycles if they did not show progressive disease (PD) and had no grade 4 hematological toxicities for more than one week. Additionally, if rapid clearance of the antibody from the circulation and high liver, spleen and bone marrow uptake due to HACA formation was detected on whole body planar images on day 2-4, patients were excluded from further RIT cycles. Retreatment followed 12 weeks after the previous therapeutic infusion at 75% of the previous activity dose. Patients were monitored for 14 weeks for hematologic toxicity. CT scanning to evaluate response according to the RECIST v.1.1 criteria was performed at baseline and 12 weeks after each RIT cycle.

## Results

Interim analysis of the therapeutic efficacy of a single infusion of $^{177}\text{Lu}$-girentuximab at the MTD of the phase I clinical trial [17] in the first 6 patients of this phase II study showed disease stabilization in 3 of 6 patients (response rate = 0.5). Because the response rate was above the predetermined cut-off of 0.25 and no prolonged grade 4 hematological toxicity was observed in these patients, recruitment was continued. A total of 14 evaluable patients will be included. Currently, data of 8 patients who received at least one infusion of $^{177}\text{Lu}$-girentuximab are available and are reported here.

In total, 9 adult patients with metastatic ccRCC were enrolled between August 2011 and August 2013. One patient was excluded from the study, because known ccRCC
lesions did not show any targeting at diagnostic imaging 6 days post injection of $^{111}$In-girentuximab. Histopathological re-examination of the original surgical specimen revealed a non-CAIX expressing ccRCC. In the other 8 patients (4 men and 4 women; median age: 71.3 year; range: 56.0–75.1 years) accumulation of $^{111}$In-girentuximab was observed in at least one of the suspect lesions (Figure 1). Patient characteristics are listed in Table 2. Seven out of 8 patients had a favorable prognosis and one patient had an intermediate prognosis according to the Memorial Sloan-Kettering Cancer Center (MSKCC) prognostic scoring system [26]. Two patients had received prior systemic treatment with VEGF-TKIs or Interferon-α (INF-α) and two patients had been subjected to local therapies (either metastasectomy or external beam radiotherapy).

Efficacy

Five out of 8 patients (63%) showed stabilization of progressive disease after the first treatment cycle (Table 2). Tumor response of the target lesions after RIT 1 is shown in Figure 2. The mean growth rate of the target lesions decreased from 18% to 10%, although large differences were observed amongst patients. Three out (#1, #4 and #5) of the 5 patients with SD after RIT 1 were eligible for a second therapeutic
**Table 2.** Patient characteristics

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Age at study entry (years)</th>
<th>Age at diagnosis (years)</th>
<th>Sex</th>
<th>Prior therapy</th>
<th>Prognostic group</th>
<th>Activity</th>
<th>Toxicity grade</th>
<th>Response after</th>
<th>PFS (months)</th>
<th>OS (months)</th>
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<tbody>
<tr>
<td>1</td>
<td>72</td>
<td>59</td>
<td>M</td>
<td>EBRT</td>
<td>Favorable</td>
<td>4857</td>
<td>3635</td>
<td>–</td>
<td>0</td>
<td>4</td>
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<tr>
<td>2</td>
<td>75</td>
<td>67</td>
<td>F</td>
<td>Sun, Paz</td>
<td>Favorable</td>
<td>4068</td>
<td>–</td>
<td>4</td>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>71</td>
<td>63</td>
<td>M</td>
<td>–</td>
<td>Favorable</td>
<td>5020</td>
<td>–</td>
<td>4</td>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>73</td>
<td>68</td>
<td>F</td>
<td>–</td>
<td>Intermediate</td>
<td>5793</td>
<td>3202²</td>
<td>–</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>56</td>
<td>51</td>
<td>F</td>
<td>Metastasectomy</td>
<td>Favorable</td>
<td>4291</td>
<td>3091</td>
<td>–</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>63</td>
<td>61</td>
<td>M</td>
<td>–</td>
<td>Favorable</td>
<td>5695</td>
<td>–</td>
<td>3</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>73</td>
<td>70</td>
<td>M</td>
<td>–</td>
<td>Favorable</td>
<td>5533</td>
<td>–</td>
<td>4</td>
<td>3</td>
<td>–</td>
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<tr>
<td>8</td>
<td>58</td>
<td>56</td>
<td>F</td>
<td>INF-α</td>
<td>Favorable</td>
<td>4333</td>
<td>–</td>
<td>4</td>
<td>4</td>
<td>–</td>
</tr>
</tbody>
</table>

MSKCC = Memorial Sloan-Kettering Cancer Center score [26]; RIT = radioimmunotherapy; L = leucocytes; T = thrombocytes; Lu = lutetium; cG250 = girentuximab; CTCAE = Common Terminology Criteria Adverse Events; RECIST = Response Evaluation Criteria in Solid Tumors; PFS = progression-free survival; OS = overall survival; PD = progressive disease; SD = stable disease; EBRT = external-beam radiation therapy; Sun = sunitinib; Paz = pazopanib

1 Patient #2 died of a hemorrhagic cerebrovascular accident 12 weeks after the first RIT unrelated to the study drug, before response evaluation was possible.
2 Due to suboptimal labeling efficiency, approximately 75% of the intended dose was administered in RIT2.
3 Patients #4 and #5 showed stable disease after two cycles, but were excluded from further treatment due to prolonged myelotoxicity.
4 Patients #7 and #8 showed stabilization of disease after one cycle, but were excluded from further treatment due to the grade 4 or prolonged myelotoxicity.
infusion. Disease progression was observed in patient #1 after the second RIT, whereas durable responses were observed in patients #4 and #5. These patients were not eligible for a third RIT due to slow recovery of myelotoxicity. Patient #8 showed SD after the first RIT, but was not eligible for retreatment because of prolonged grade 4 myelotoxicity. Patients #2, #3 and #7 showed progression of disease during the first RIT cycle. Patient #2 died after a hemorrhagic cerebrovascular accident 12 weeks after the first RIT, one week before response evaluation was planned. CT imaging of the cerebrum revealed that the hemorrhage was caused by a previously undetected cerebral metastases. Because this patient had recovered from hematological toxicity, it was concluded that this event was unrelated to the study drug. Patient #3 had

![Figure 2. Tumor growth of the target lesions after RIT 1 per patient and the mean of 8 patients according to RECIST criteria v.1.1.](image-url)
rapid progression of liver metastases during the first RIT cycle and was therefore excluded for retreatment. In patient #7, CT imaging of chest and abdomen showed stabilization of the target lesions, however, a new bone lesion in the right shoulder was detected with $^{111}$In-girentuximab scintigraphy at the start of the second RIT cycle (Figure 3). Conventional radiological studies confirmed this observation and the patient was subsequently eligible for additional $^{177}$Lu-girentuximab infusions.

For all patients, the available PFS and OS intervals are listed in Table 2. The median PFS after the first infusion of $^{177}$Lu-girentuximab was 6.9 months. Because the numbers are small and follow-up of several patients is ongoing, no additional statistical analyses were performed.

*Figure 3.* $^{111}$In-girentuximab imaging at the start of RIT 1 and RIT 2 and MRI image of right shoulder during RIT 2. Despite stabilization of disease in the chest and abdomen after RIT 1 in patient #7, a new bone lesion in the right shoulder detected with $^{111}$In-girentuximab scintigraphy at the start of the second RIT cycle indicated progressive disease. Magnetic Resonance Imaging (MRI) of the right shoulder confirmed this observation and the patient was therefore excluded from the study.
Toxicity

All patients experienced grade 3-4 thrombocytopenia in the first RIT cycle, with the exception of patient #6 (grade 2). The nadir of the platelet count was at median of 5 weeks post injection of $^{177}$Lu-girentuximab (Figure 4). Generally, patients recovered from grade 3 or higher thrombocytopenia in 2-3 weeks. In total, 3 patients developed a severe thrombocytopenia requiring at least one thrombocyte infusion. Absolute platelet counts after RIT 1 were $> 150 \times 10^9$/l in all but 1 patient, although none of the patients reached their pre-RIT thrombocyte levels. In the 3 patients receiving a second therapeutic injection, the hematological toxicity was less severe than in the first RIT cycle, however, slow recovery from myelotoxicity precluded a third treatment cycle in 2 patients. Transient grade 2-4 leucocytopenia was observed in 7 patients, with the nadir at a median of 6 weeks after the therapeutic injection (Figure 4). Three patients required hospitalization due to febrile neutropenia.

Apart from the myelotoxicity, treatment with $^{177}$Lu-girentuximab was generally well tolerated, as 7 patients experienced only mild (grade 1-2) fatigue and nausea. No allergic reactions due to the girentuximab infusions were observed.

Discussion

Interim analysis of the currently available data from 8 patients indicate that $^{177}$Lu-girentuximab RIT resulted in stabilization of previously progressive disease in 5 out of 8 patients with advanced ccRCC. The mean growth rate of the target lesions decreased after the first RIT cycle (Figure 2), although the overall results are clearly influenced by several outliers. Six additional patients who will receive at least one $^{177}$Lu-girentuximab infusion will be recruited to complete the study.
Figure 4. Hematological toxicity during RIT 1. Thrombo- and leucocyte counts are presented as mean ± standard error of the mean (s.e.m.).
RIT with $^{177}$Lu-girentuximab resulted in few side effects, although reversible grade 3-4 hematological toxicity was observed in all patients (Table 2), which is in concordance with the results of RIT treatment in patients with other solid tumors [25, 27]. In the 3 patients treated with two RIT cycles, successive injections with $^{177}$Lu-girentuximab at 75% of the previous activity dose did not result in higher toxicity levels. The prevalence of grade 3-4 hematological toxicity seems higher than in the patients treated at the same dose level (2405 MBq/m$^2$) in the phase I trial. To allow subsequent treatment with TKIs (which can also cause hematological toxicity), RIT was discontinued in 4 patients who experienced prolonged thrombo- or leucocytopenia. Because of the relatively high incidence of grade 3-4 hematological toxicity, the currently used dose of 2405 MBq/m$^2$ may have to be adjusted to allow all patients to complete the three treatment cycles in case of disease stabilization.

The results of the current study are encouraging, however, implementation of girentuximab-based RIT into clinical practice is not yet possible. At present, one of the biggest challenges is to identify patients who will benefit most from RIT. Previous studies indicate that RIT is mainly suitable for treatment of small volume disease or possibly as adjuvant treatment in selected cases [13, 28]. Although our study was designed to evaluate the efficacy of $^{177}$Lu-girentuximab RIT in patients with metastatic ccRCC, it would also be worthwhile to test $^{177}$Lu-girentuximab RIT as adjuvant therapy in the future. In that case, the activity dose of 2405 MBq/m$^2$ may have to be lowered to prevent severe myelotoxicity in an adjuvant setting.

Besides optimization of the timing of RIT in the management of ccRCC, personalized dosing based on dosimetric analysis of the data acquired with the pre-treatment $^{111}$In-girentuximab imaging is likely to improve $^{177}$Lu-girentuximab RIT. Previous studies have clearly shown the correlation between red bone marrow dose and hematological toxicity, and indicate that diagnostic data can be used to accurately
predict absorbed doses and myelotoxicity of girentuximab-mediated RIT [29, 30]. This approach is of particular interest, as the trade-off between efficacy and toxicity can be tailored to the individual patient. In the near future, the Positron Emission Tomography (PET) tracer zirconium-89-girentuximab will be evaluated in ccRCC patients. Preclinical data indicate superior imaging characteristics compared to both the $^{111}$In-labeled and the radioiodinated antibody [17, 31], and PET will allow more accurate quantification of activity doses in the relevant tissues. Usage of this tracer might improve the identification of patients who benefit most from $^{177}$Lu-girentuximab infusions and will allow patient-tailored dosing of RIT.

Last but not least, an important step to successfully implement girentuximab-based RIT is to optimize combinations with other treatment modalities such as TKIs, the current standard of care in metastatic ccRCC. As we recently reported, treatment with TKIs has a profound effect on the uptake of $^{111}$In-girentuximab in ccRCC lesions [32]. Data from the latter study indicate that the tumor targeting of girentuximab-based RIT is severely hampered if given during TKI treatment and that RIT should preferably be given either before TKI treatment or after cessation of the TKI treatment. Further studies are warranted to evaluate the duration of this TKI-induced effect and could help to improve treatment strategies for metastatic ccRCC.

In conclusion, the interim results of the currently available data of this ongoing phase II RIT trial with $^{177}$Lu-girentuximab are encouraging in terms of clinical response in patients with progressive metastatic ccRCC. Apart from transient myelotoxicity, the toxicity profile of $^{177}$Lu-girentuximab RIT seems to be mild. Final analyses of this phase II trial will be performed after inclusion of 6 additional patients and will hopefully shed more light on the therapeutic efficacy and safety of this treatment modality in patients with metastatic ccRCC.
References


Chapter 9

General discussion and future perspectives
General discussion and future perspectives

This thesis describes several studies regarding the detection and treatment of clear cell renal cell carcinoma (ccRCC) with monoclonal antibody G250/girentuximab.

Radioimmunodetection

Detection of ccRCC with radiolabeled girentuximab clearly holds promise for the future because of its high sensitivity and specificity in detecting both primary ccRCC lesions and disseminated disease. Chapter 3 describes our experience with indium-111 ($^{111}$In) labeled girentuximab as a non-invasive diagnostic tool to detect ccRCC. We found that the presence of ccRCC is highly likely in case of a positive $^{111}$In-girentuximab immunoSPECT. However, when no accumulation of the radiolabeled antibody is observed in the target lesions, other, non-ccRCC malignancies cannot be ruled out due to the current lack of specific tracers for these subtypes. In case of a negative $^{111}$In-girentuximab immunoSPECT in patients presenting with a small renal mass, active surveillance or biopsy is warranted to further assess the nature of the suspect lesions.

In addition to our studies focusing on the diagnostic value of $^{111}$In-labeled girentuximab in ccRCC, extensive experience has been gathered with Positron Emission Tomography (PET) tracer iodine-124 ($^{124}$I)-girentuximab in other centers [1, 2]. An important difference between these two girentuximab conjugates is that $^{111}$In is a residualizing radiometal that will be retained in the cell after internalization, whereas radioiodinated girentuximab is rapidly excreted after internalization, leading to a markedly reduced tumor visualization [3, 4]. Compared to $^{124}$I-girentuximab, $^{111}$In-girentuximab is easier to produce as an off-the-shelf agent because it does not require specialized equipment or additional purification steps after the labeling procedure. However, clear advantages of $^{124}$I-girentuximab immunoPET compared to $^{111}$In-girentuximab
immunoSPECT are the superior spatial resolution and the more rapid image acquisition of PET and the more accurate quantitative analysis of the PET images.

Although it is likely that the use of girentuximab-based imaging will increase in the near future, it is yet to be determined whether it also can be used for treatment monitoring or the selection of patients. Several clinical trials regarding this topic are currently ongoing or planned. One particularly interesting trial that will be initiated in the near future aims to use molecular imaging to select patients for the currently available treatment modalities for ccRCC. This will potentially allow more effective, better tolerated and more cost-effective treatment strategies. Moreover, this trial will be using the new PET tracer zirconium-89 ($^{89}$Zr)-girentuximab. $^{89}$Zr is a long-lived PET isotope (t½ = 3.27 days) and is a residualizing radiometal that can serve as a surrogate to predict the in vivo distribution and tumor targeting of the antibody labeled with yttrium-90 or lutetium-177 ($^{177}$Lu). Because $^{89}$Zr-girentuximab is a residualizing PET tracer, it has important advantages over both $^{111}$In- and $^{124}$I-girentuximab. As preclinical data already suggest superior imaging characteristics of $^{89}$Zr-girentuximab compared to both the $^{111}$In [5] and $^{124}$I-labeled [6, 7] antibody, it will be very interesting to see whether this new tracer indeed represents the best of both worlds.

**Optical imaging of ccRCC**

In addition to improved detection and (re)staging, patient management can potentially be further improved by enhanced intraoperative detection of CAIX-expressing lesions with girentuximab-mediated near infrared fluorescence imaging. However, tissue penetration of the emitted light is limited, potentially hampering detection of lesions. This problem may be overcome by a dual-label strategy using antibodies that combine fluorescence imaging with radionuclide detection. Such a combination enables enhanced detection of lesions by the radioactive signal, which provides
sufficient tissue penetration and allows for real-time optical imaging. The results of the preclinical studies described in chapter 4 and 5 unequivocally demonstrate that both pre- and intraoperative imaging of ccRCC lesions might be feasible with dual-labeled girentuximab. Although the use of interventional molecular imaging techniques may very well lead to new surgical treatment paradigms for ccRCC, there are still several hurdles to overcome before these techniques can be implemented in the operating theatre. Therefore, more studies are warranted to achieve the clinical translation of this imaging approach. Currently, a phase I clinical trial with dual-label antibody preparation $^{111}$In-girentuximab-IRDye800CW is in preparation. The main objective of this trial will be to assess the safety and feasibility of dual modality imaging with this dual-labeled antibody in patients with a primary renal tumor suspect for ccRCC. If it is indeed possible to detect ccRCC lesions with both modalities in the clinical setting, additional studies are warranted to evaluate the validity and cost-effectiveness of this approach.

Prospects in girentuximab-mediated radioimmunotherapy

Apart from the advances in radioimmunodetection, substantial progress has been made with girentuximab-based radioimmunotherapy (RIT) strategies in ccRCC in the last decade. In chapter 8, the encouraging, preliminary results of the ongoing phase II RIT trial indicate that RIT might someday be an important component in the treatment of ccRCC. However, before girentuximab-based RIT can be implemented in clinical practice, several issues need to be resolved. Although $^{177}$Lu-girentuximab RIT is generally well tolerated, some patients experience prolonged bone marrow suppression, which can result in the need for repeated thrombocyte infusions or hospital admission in case of leucopenia. In addition, sustained myelotoxicity potentially hampers future treatment with tyrosine kinase inhibitors (TKIs).
A logical intervention to avoid prolonged myelotoxicity would be to lower the administered activity dose during the three treatment cycles. Although reduction of, for instance, 20% would better circumvent bone marrow suppression according to previous results [8], this could potentially lead to lower efficacy of the treatment, which is clearly undesirable. It is expected that advances in dosimetric analysis will contribute to the improvement of patient-specific dosing in RIT, because the trade-off between efficacy and toxicity can then be better tailored to the individual patient.

Another important challenge is the identification of patients that might benefit most from RIT. Previous studies indicate that RIT is mainly suitable for treatment of small volume disease or possibly as adjuvant treatment in selected cases [9, 10]. Experiences gathered in the ARISER trial that showed that prevention of disease recurrence by girentuximab depended on CAIX expression levels [11]. It is highly likely that, in future, tumor-specific characteristics such as CAIX expression will be used for patient stratification. Although histopathological examination of biopsies or surgical specimens currently is the gold standard for treatment decisions, molecular imaging may replace these in the future. As mentioned earlier, the ongoing clinical trials with $^{89}$Zr-girentuximab should provide critical evidence in the upcoming years.

Another challenge to successfully implement girentuximab-based RIT in patient care is optimizing the combination of this therapeutic modality with the current standard of care in metastatic ccRCC, namely treatment with tyrosine kinase inhibitors (TKIs). As described in chapter 7, treatment with TKI sorafenib had a profound effect on the uptake of $^{111}$In-girentuximab in ccRCC lesions. These data indicate that the effect of girentuximab-based RIT would be severely hampered if given during TKI treatment. As a result, RIT should preferably be given either before TKI treatment or after cessation of the TKI treatment. Because of the maximal efficacy of RIT in small volume disease [10] and the mild toxicity profile compared to TKI treatment,
it would probably be most advantageous to start with RIT prior to TKI treatment in case of metastatic ccRCC. Carefully designed clinical trials regarding optimization of treatment combinations will hopefully lead to better patient care in the near future.
References


Approximately 2200 new patients are diagnosed with renal cell carcinoma (RCC) in the Netherlands each year. Due to the increased use of conventional imaging techniques such as ultrasound, Computed Tomography (CT) or Magnetic Resonance Imaging (MRI), RCC is found more often in an earlier stage. However, a major drawback of conventional radiological imaging techniques is that they cannot reliably distinguish benign solid lesions from RCC, which often poses clinicians for a diagnostic dilemma. Adequate characterization of these lesions based on imaging is essential to avoid invasive biopsies and superfluous surgery, but more importantly to not overlook a potentially lethal disease.

The poor diagnostic performance of conventional radiological imaging techniques has led to the search for molecular imaging techniques that target disease-specific features. In 1986, the monoclonal antibody (mAb) G250 (the chimeric variant of the mAb was later denominated girentuximab) that recognized an antigen abundantly expressed on RCC tumor cells was first described. The unknown antigen was later identified as a member of the Carbonic Anhydrase family and subsequently denominated Carbonic Anhydrase IX (CAIX). The antigen is ubiquitously expressed in clear cell RCC (ccRCC), the most predominant subtype of RCC, which accounts for approximately 85% of the cases. CAIX is overexpressed in up to 94% of the ccRCC cases and is not expressed in normal kidney tissue or renal cysts. This high and specific expression of the antigen allows for both antibody-mediated imaging and therapy. A detailed overview of important preclinical and clinical studies is summarized in chapter 2. The aim of this thesis was to further optimize the detection and treatment of ccRCC using radiolabeled mAb G250/girentuximab.

In chapter 3, the use of indium-111 ($^{111}$In)-labeled girentuximab Single Photon Emission Computed Tomography (SPECT) as a diagnostic tool in ccRCC patients is evaluated. This chapter describes a series of both patients with primary tumors of
unknown origin and patients with a history of ccRCC presenting with lesions suspect for metastases on follow-up imaging. All malignant ccRCC lesions were visualized specifically with $^{111}$In-girentuximab SPECT, whereas benign lesions were not. Moreover, $^{111}$In-girentuximab imaging was found to be a helpful, non-invasive tool for clinical decision making in patients with a history of ccRCC presenting with lesions suspect for metastases, particularly in cases with inconclusive conventional imaging or biopsies.

In addition to preoperative radionuclide imaging, radiotracers are frequently used during surgical procedures. By now, sentinel-node procedures have become a well-established technology in several tumor types. Although this technique may help guide the surgeon to the lymph node of interest, it cannot provide a precise delineation of tumor lesions or resection margins. The addition of a near infrared (NIR) fluorescent label could help to overcome this limitation. Because surgery for ccRCC could potentially benefit from fluorescence imaging by facilitating intra-operative detection tumor lesions, mAb girentuximab was conjugated with optical dye IRDye800CW and tested in several animal experiments.

In chapter 4, the feasibility to detect ccRCC tumors with fluorescence imaging using G250-IRDye800CW was studied in mice with subcutaneous growing ccRCC xenografts. In this study, the antibody was conjugated with IRDye800CW and radioiodinated with iodine-125 ($^{125}$I) to allow SPECT imaging as a reference. Subcutaneous CAIX-expressing ccRCC xenografts were clearly visualized with fluorescence imaging using $^{125}$I-G250-IRDye800CW and showed good concordance with the SPECT images. Ex vivo biodistribution studies confirmed the high and specific targeting of the antibody preparation in the ccRCC tumors.

To study the potential of fluorescence imaging of CAIX-expressing tumors more extensively, this approach was tested in a more advanced ccRCC model.
with intraperitoneal (i.p.) growing tumor nodules (chapter 5). In this study, the antibody-dye conjugate was labeled with $^{111}$In, to allow microSPECT imaging. Again, SPECT and fluorescence images showed clear delineation of the i.p. CAIX-expressing ccRCC lesions. Moreover, no residual tumor was detected with fluorescence imaging or macroscopically after the imaged-guided surgery, indicating that pre- and intraoperative detection of CAIX-expressing lesions might be feasible with dual-label G250.

Apart from the diagnostic issues in ccRCC, there is an unmet need for improved treatment of advanced disease. Over the last decade, the development of agents targeting the vascular endothelial growth factor (VEGF) pathway like bevacizumab (with Interferon-$\alpha$), tyrosine kinase inhibitors (TKIs) sunitinib, pazopanib, axitinib, and sorafenib and mammalian target of rapamycin (mTOR) inhibitors like temsirolimus and everolimus have marked a new era in the treatment of metastatic ccRCC. Although these targeted therapies have demonstrated good clinical efficacy in terms of progression free survival (PFS), treatment with these agents is chronic and side effects occur frequently and can be severe. The search for novel systemic treatment strategies with less toxicity and significant anti-tumor effect has led to girentuximab-based radioimmunotherapy (RIT). Chapter 2 provides an overview of efforts made to develop successful RIT strategies for metastatic ccRCC, eventually leading to using lutetium-177 ($^{177}$Lu) labeled girentuximab.

In chapter 6, the potential of of radioimmunodetection and RIT with radiolabeled G250 was evaluated in a model with i.p. growing ccRCC xenografts. First, the optimal antibody dose was determined in a protein dose escalation study. This antibody dose was used in a subsequent RIT study, where the efficacy of a single injection of $^{177}$Lu-G250 was evaluated. Treatment with $^{177}$Lu-G250 resulted in significantly improved median survival compared to the mice in the control groups, indicating that this RIT
was also effective in this model that mimics metastasized ccRCC. The model proved reliable and is suitable for further optimizing RIT and combinations of RIT and other treatment modalities in the future.

Besides preclinical studies focusing on optimizing $^{177}$Lu-girentuximab RIT, $^{177}$Lu-girentuximab has been used in a phase I trial. In chapter 8, the preliminary results of an ongoing phase II RIT trial with $^{177}$Lu-girentuximab are reported. The primary endpoint of this study is to determine the efficacy of $^{177}$Lu-girentuximab in terms of tumor response using the Response Evaluation Criteria In Solid Tumors (RECIST). Secondary objectives are to determine the PFS, overall survival, and to evaluate treatment’s toxicity.

Patients with advanced progressive ccRCC are treated with a maximum of three consecutive RIT infusions at the predetermined maximum tolerated dose of 2405 MBq/m$^2$ in the first cycle. If an objective response (defined as at least stable disease) is seen after the first treatment cycle, patients are eligible for additional treatment cycles at 75% of the activity dose of the previous treatment cycle. To ensure antibody accumulation in the metastatic lesions, patients receive a diagnostic infusion with $^{111}$In-girentuximab before the therapeutic infusion with $^{177}$Lu-girentuximab at the start of each treatment cycle.

From August 2011 to August 2013, 9 patients enrolled in the study. Because no targeting of $^{111}$In-girentuximab was observed in one of the patients, a total of 8 patients with metastatic ccRCC were eligible for treatment with $^{177}$Lu-girentuximab. Objective responses after one treatment cycle were observed in 5 out of 8 patients. Of these five patients, two were not eligible for a second therapeutic injection due to prolonged hematological toxicity. Two patients with objective responses after one cycle showed a prolonged response after the second $^{177}$Lu-girentuximab
infusion, while the other patient showed disease progression after 6 months. Treatment with $^{177}$Lu-girentuximab was generally well tolerated, but resulted in transient grade 3-4 thrombocytopenia and leukopenia in all patients. Most patients recovered fully within several weeks, but in some patients prolonged bone marrow suppression was observed, resulting in exclusion for additional treatment cycles.

As the evidence builds that $^{177}$Lu-girentuximab has therapeutic efficacy in the majority of patients with advanced ccRCC, the question arises whether this approach can be combined with the approved therapeutic agents in ccRCC, more specifically TKIs. In chapter 7, the effect of widely used TKI sorafenib on the tumor uptake of mAb girentuximab was assessed in patients presenting with a renal mass of unknown origin. $^{111}$In-girentuximab imaging was performed twice in 10 patients scheduled for (partial) nephrectomy. In between scans, patients were treated with sorafenib for either 1 or 4 weeks. Neoadjuvant treatment with sorafenib resulted in a marked decrease of $^{111}$In-girentuximab uptake in the ccRCC lesions in these patients, especially in the group treated during 4 weeks.

The results of this study clearly show that the effect of antibody-mediated treatment modalities would be profoundly hampered when given during TKI treatment and therefore has major implications for future therapeutic regimens combining both treatments.

In summary, the studies described in this thesis show that girentuximab-mediated detection has great potential for both pre- and intraoperative diagnosis of ccRCC. In addition, current results indicate that $^{177}$Lu-girentuximab radioimmunotherapy yields clinical benefit in the majority of patients with previously progressive ccRCC, although combinations with other treatment modalities should be carefully chosen.
Samenvatting

Jaarlijks wordt bij ongeveer 2200 patiënten in Nederland nierkanker vastgesteld. Gelukkig wordt deze ziekte door het toegenomen gebruik van conventionele radiologische technieken zoals echografie, CT en MRI tegenwoordig vaker in een vroeger stadium gevonden.

Een nadeel van bovengenoemde technieken is echter dat zij vaak geen onderscheid kunnen maken tussen goedaardige en kwaadaardige tumoren van de nier. Dit laatste is echter van groot belang voor de behandeldend arts: deze wil voorkomen dat patiënten onnodige biopsieën of overbodige operaties ondergaan, maar patiënten met een potentieel dodelijke ziekte dienen uiteraard adequaat behandeld te worden.

De zoektocht naar technieken die beter in staat zijn om onderscheid te maken tussen goedaardige en kwaadaardige tumoren, hebben geleid tot nieuwe beeldvormende technieken die gebruik maken van ziektespecifieke eigenschappen. Midden jaren ’80 werd een monoklonaal antilichaam gevonden dat zeer specifiek gericht was tegen een speciek type nierkanker, het heldercellig niercelcarcinoom (Engels: clear cell renal cell carcinoma of ccRCC). Het antilichaam werd in de eerste instantie G250 genoemd en later girentuximab. Enkele jaren later werd het eiwit (=antigeen) gevonden waaraan G250 bindt. Dit antigeen, Carbonic Anhydrase IX (CAIX) genaamd, bleek in zeer hoge mate tot expressie te komen bij ccRCC tumoren, maar niet in normaal nierweefsel of niercysten. Daarnaast komt dit antigeen slechts in zeer beperkte mate elders in het lichaam voor.

Heldercellige nier tumoren vertegenwoordigen ongeveer 85% van alle kwaadaardige tumoren van de nier en overexpressie van CAIX wordt gevonden in ongeveer 94% van de heldercellige tumoren. Door hoge en specifieke expressie van CAIX in ccRCC tumoren, is dit antigeen bij uitstek geschikt voor doelgerichte diagnostiek en
therapie met behulp van het G250 antilichaam. In hoofdstuk 2 wordt een uitgebreide beschrijving gegeven van de belangrijkste preklinische en klinische studies met G250/ girentuximab tot op heden.

In dit proefschrift is een aantal klinisch relevante vragen met betrekking tot het gebruik van G250/girentuximab onderzocht. Allereerst is er gekeken naar de mogelijkheid om met radioactief gelabeld girentuximab ccRCC tumoren (preoperatief) te detecteren. Ten tweede is er gezocht naar nieuwe technieken om niet-tumoren en uitzaaiingen van nierkanker te visualiseren met behulp van fluorescentie. Als laatste worden de voorlopige resultaten van de nog lopende radioimmunotherapie studie met girentuximab en de implementatie van deze nieuwe therapievorm in de kliniek besproken.

Hoofdstuk 3 beschrijft het gebruik van indium-111 (\(^{111}\text{In}\))-gelabeld girentuximab als diagnostisch hulpmiddel. \(^{111}\text{In}\)-girentuximab is een radioactieve tracer die gedetecteerd kan worden met een Single Photon Emission Computed Tomography (SPECT) camera. Zo kunnen ccRCC tumoren op een niet-invasieve manier in beeld worden gebracht. In deze studie werden zowel patiënten met een nier tumor van onbekende origine, als patiënten met ccRCC in de voorgeschiedenis waarbij er op basis van conventionele beeldvorming verdenking is op uitzaaiingen, met \(^{111}\text{In}\)-girentuximab SPECT onderzocht. Alle heldercellige niet-tumoren werden gedetecteerd met de \(^{111}\text{In}\)-girentuximab SPECT scan, terwijl goedaardige tumoren niet aankleurden. Daarnaast kon bij een groot aantal patiënten met verdenking op uitgezaaide nierkanker uitsluitso gegeven worden over de aard van de afwijkingen.

Naast het gebruik bij verschillende (preoperatieve) nucleaire beeldvormende technieken, worden radiotracers veelvuldig ingezet bij schildwachtklierprocedures (o.a. bij het melanoom en bij borstkanker). Hoewel de chirurg bij deze procedures
naar de eerst-drainerende lymfeklier geleid wordt, is deze techniek niet in staat om tumoren te visualiseren of af te bakenen. Door het toevoegen van een fluorescent label aan het radioactieve girentuximab, kunnen tumoren of lymfeklieren mogelijk beter gedetecteerd worden. Omdat de chirurgische behandeling van ccRCC wellicht ook verbeterd zou kunnen worden deze technieken, werden een aantal preklinische experimenten uitgevoerd waarbij G250 met zowel een radioactieve als een fluorescente stof werd gelabeld.

Hoofdstuk 4 beschrijft experimenten in muizen met subcutane ccRCC tumoren, waarbij onderzocht is of de tumoren afgebeeld kunnen worden met fluorescentie technieken. In dit experiment werd G250 gelabeld werd met de fluorescerende stof IRDye800CW. Daarnaast werd G250 gemarkeerd met radioactief jodium, zodat het tevens mogelijk was om de tumoren met een SPECT camera af te beelden. De tumoren konden met zowel fluorescentie als met SPECT beeldvorming duidelijk afgebeeld worden, waarbij er grote overlap was tussen beide technieken.

Vervolgens werden deze fluorescentie technieken getest in een meer geavanceerd diermodel, waarbij de ccRCC tumoren in de buikholte van de muizen groeiden (hoofdstuk 5). Wederom werd G250 dubbel gelabeld met zowel een radioactieve als een fluorescerende stof. Ook in dit model bleek het mogelijk om de tumoren met beide technieken in beeld te brengen. Bovendien kon er geen resttumor gedetecteerd worden na verwijdering van de tumoren die gevonden waren met behulp van de fluorescentie beeldvorming.

Beide studies tonen aan dat het mogelijk is om met behulp van doelgerichte fluorescentie technieken ccRCC tumoren in beeld te brengen, hetgeen mogelijk kan leiden tot gebruik van deze technieken gedurende operaties vanwege heldercellige nier tumoren of uitzaaiingen van deze ziekte.
Naast de beperkingen in de detectie van niertumoren met de huidige beeldvormende technieken, is er ook nog steeds een grote behoefte aan nieuwe behandelopties voor deze ziekte. Hoewel er de afgelopen jaren goede resultaten zijn geboekt met een aantal medicijnen die de aanmaak van nieuwe bloedvaten in de tumor remmen, is de behandeling met deze zogenaamde angiogeneseremmers (Grieks: angio=bloedvat, genesis=ontstaan) chronisch en helaas niet zonder bijwerkingen.

Ook op dit gebied zou doelgerichte interventie met G250/girentuximab een rol kunnen spelen. Door het antilichaam te labelen met een hoge dosis van een radioactieve stof die β-straling uitzendt (bijvoorbeeld met radionucliden als yttrium-90, jodium-131 of lutetium-177 (\(^{177}\text{Lu}\))) kan de straling specifiek in de ccRCC afgeleverd worden en kunnen de tumorcellen gedood worden. Deze therapievorm wordt radioimmunotherapie genoemd.

In hoofdstuk 2 wordt een uitgebreide beschrijving van de belangrijkste preklinische en klinische therapie studies met G250/girentuximab gegeven, welke uiteindelijk hebben geleid tot studies met \(^{177}\text{Lu}\) gelabeled girentuximab.

Hoofdstuk 6 beschrijft de optimalisatie van \(^{177}\text{Lu}-\text{G250}\) radioimmunotherapie in een muismodel waarbij de ccRCC tumoren in de buikholte van de muizen groeiden. Allereerst werd de optimale antilichaam dosis in dit model bepaald, waarna deze dosis werd gebruikt in een vervolg studie waarbij het effect van één injectie met \(^{177}\text{Lu}-\text{G250}\) werd geëvalueerd. De mediane overleving van de muizen behandeld met \(^{177}\text{Lu}-\text{G250}\) was significant langer dan de muizen in de controle groepen. In de toekomst zouden in dit model ook combinaties van radioimmunotherapie en andere therapievormen getest kunnen worden.

Naast preklinische studies met \(^{177}\text{Lu}\)-girentuximab, is radioimmunotherapie ook al in de
klinische setting geëvalueerd. In hoofdstuk 8 worden de voorlopige resultaten van een nog lopende fase II radioimmunotherapie studie met $^{177}$Lu-girentuximab in patiënten met gemetastaseerd ccRCC beschreven. Het primaire eindpunt van deze studie is de effectiviteit van $^{177}$Lu-girentuximab volgens de zogenaamde ‘Response Evaluation Criteria In Solid Tumors’. Daarnaast zal de progressievrije overleving, de totale overleving en de toxiciteit van deze therapie in deze patiënten worden geëvalueerd.

Patiënten met progressieve, uitgezaaide ccRCC worden behandeld met maximaal drie opeenvolgende therapeutische injecties met $^{177}$Lu-girentuximab. Tijdens de eerste cyclus worden patiënten behandeld met op een dosisniveau van 2405 MBq/m$^2$. De veiligheid van deze dosis radioactiviteit werd reeds in voorgaande studies vastgesteld. Indien de eerste therapeutische injectie effectief is (gedefinieerd als afname van de uitzaaiingen of stabiele ziekte na drie maanden), kunnen patiënten in aanmerking komen voor een tweede of derde behandelingcyclus. Het dosisniveau van deze vervolgcyclus is telkens 75% van de voorgaande dosis. Om er zeker van te zijn dat het antilichaam daadwerkelijk opgenomen wordt in de uitzaaiingen, krijgen patiënten voorafgaand aan de therapeutische injectie altijd eerst een diagnostische injectie met $^{111}$In-girentuximab.

Van augustus 2011 tot augustus 2013 werden negen patiënten geïncludeerd in de studie. Na de diagnostische injectie werd er bij één patiënt geen opname van het $^{111}$In-gelabelde girentuximab gedetecteerd in de uitzaaiingen, waarop deze patiënt niet in aanmerking kwam voor de therapeutische injectie. De overige acht patiënten werden wel behandeld met $^{177}$Lu-girentuximab. Bij vijf van de acht patiënten trad stabilisatie van de ziekte op, al konden twee van hen geen tweede injectie krijgen vanwege beenmergtoxiciteit. De overige drie patiënten ontvingen een tweede therapeutische injectie, hetgeen opnieuw resulteerde in stabilisatie van de ziekte bij twee patiënten.
Samenvatting

De radioimmunotherapie werd over het algemeen zeer goed verdragen, maar resulteerde in ernstige, tijdelijke onderdrukking van de beenmergfunctie in vrijwel alle patiënten. In enkele gevallen was het herstel van het beenmerg echter dusdanig traag dat aanvullende behandeling met $^{177}$Lu-girentuximab – mede met het oog op eventuele toekomstige behandelingen - niet verantwoord werd geacht.

Nu er in toenemende mate aanwijzingen gevonden worden dat $^{177}$Lu-girentuximab radioimmunotherapie therapeutisch effectief kan zijn in patiënten met uitgezaaide nierkanker, rijst de vraag of deze therapievorm gecombineerd kan worden met de standaard therapie voor uitgezaaide ziekte, oftewel of radioimmunotherapie gecombineerd kan worden met behandeling met angiogeneseremmers.

In hoofdstuk 7 wordt het effect van angiogeneseremmers op de opname van het antilichaam girentuximab in tumoren beschreven. Om dit te onderzoeken, werden tien patiënten die een tumornefrectomie zouden ondergaan, twee keer geïnjecteerd met $^{111}$In-girentuximab en vervolgens gescand. Tussen deze scans werden de patiënten gedurende één of vier weken behandeld met de angiogeneseremmer sorafenib. Bij een controle groep van vijf patiënten werd alleen een scan gemaakt na toediening van $^{111}$In-girentuximab, zonder behandeling met sorafenib. Na de laatste scan werden de patiënten geopereerd en werden de veranderingen op weefselniveau en de opname $^{111}$In-girentuximab in de tumor geanalyseerd.

Behandeling met sorafenib resulteerde in een significante vermindering van de opname $^{111}$In-girentuximab in de niertumoren, met name in de groep die vier weken behandeld was met de angiogeneseremmer. Deze resultaten laten zien dat behandeling met radioimmunotherapie negatief beïnvloed wordt door gelijktijdige behandeling met angiogeneseremmers.
De studies beschreven in dit proefschrift dragen bij aan de kennis op het gebied van de pre- en intraoperatieve detectie van nierkanker en de behandeling met radioactief gelabeld G250/girentuximab. 172Lu-girentuximab radioimmunotherapie lijkt stabilisatie van progressieve, uitgezaaide nierkanker te bewerkstelligen in de meeste patiënten, hoewel combinaties met andere therapievormen zorgvuldig gekozen dienen te worden.
List of publications


Curriculum Vitae


Gedurende de onderzoekstijd werden tijdens internationale congressen verschillende prijzen gewonnen, waaronder een award voor ‘Best Fundamental Research Paper Published in European Urology in 2013’. Naast de onderzoekswerkzaamheden was hij gedurende 2,5 jaar als lid en voorzitter betrokken bij de Clinical PhD Council van het Radboudumc en is hij lid van de Stichting Wetenschappelijk Onderzoek Assistenten Heelkundige Specialismen (SWOAHS).

Sinds januari 2014 is hij in opleiding tot uroloog en in het kader van zijn vooropleiding werkt hij op de afdeling Heelkunde in het Rijnstate Ziekenhuis te Arnhem (opleider dr. M.M.P.J. Reijnen).
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