Overexpression of the epidermal growth factor receptor (EGFR) is linked to an adverse outcome in various solid tumors. Cetuximab is an EGFR inhibitor, which in combination with radiotherapy improves locoregional control and survival in a subgroup of patients with head and neck squamous cell carcinomas (HNSCCs). The aim of this study was to develop and characterize an EGFR-directed PET tracer, $^{64}$Cu-cetuximab-F(ab$'$)$_2$, to determine the systemic accessibility of EGFR. Mice with HNSCC xenografts, UT-SCC-8 ($n=6$) or UT-SCC-45 ($n=6$), were imaged 24 h post injection with $^{64}$Cu-NODAGA-cetuximab-F(ab$'$)$_2$ using PET/CT. One mouse for each tumor model was co-injected with excess unlabeled cetuximab 3 days before radiotracer injection to determine non-EGFR-mediated uptake. Ex vivo biodistribution of the tracer was determined and tumors were analyzed by autoradiography and immunohistochemistry. The SUV$_{\text{max}}$ of UT-SCC-8 tumors was higher than that of UT-SCC-45: $1.5 \pm 1.0$ and $0.8 \pm 0.2$ ($p<0.05$), respectively. $\text{SUV}_{\text{max}}$ after in vivo blocking of EGFR with cetuximab was 0.4. Immunohistochemistry showed that UT-SCC-8 had a significantly higher EGFR expression than UT-SCC-45: $0.50 \pm 0.19$ versus $0.12 \pm 0.08$ ($p<0.005$), respectively. Autoradiography indicated that $^{64}$Cu-cetuximab-F(ab$'$)$_2$ uptake correlated with EGFR expression in both tumors: $r=0.86 \pm 0.06$ (UT-SCC-8) and $0.90 \pm 0.06$ (UT-SCC-45). $^{64}$Cu-cetuximab-F(ab$'$)$_2$ is a promising PET tracer to determine expression of EGFR in vivo. Clinically, this tracer has the potential to be used to determine cetuximab targeting of tumors and possibly to non-invasively monitor the response to EGFR-inhibitor treatment. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: $^{64}$Cu; EGFR; PET/CT; cetuximab; F(ab$'$)$_2$; HNSCC

1. INTRODUCTION
The epidermal growth factor receptor (EGFR) is a transmembranous protein linked to many regulatory cellular pathways. Its overexpression and activation can have significant effects on cellular processes and are observed in many cancers (1,2). Anti-EGFR antibodies have shown clinical activity in a variety of solid tumors including head and neck, colon, non-small-cell lung and renal cell carcinomas, thereby affecting signaling pathways leading to cell cycle arrest, apoptosis and inhibition of angiogenesis and metastasis formation (3). The EGFR is overexpressed in 80–100% of head and neck squamous cell carcinomas (HNSCCs), and the monoclonal antibody cetuximab when combined with radiotherapy has proven to be clinically effective in a small subpopulation of HNSCC patients (4,5). In order to progress to individualized treatment and reduce treatment-related toxicity, patient selection is of major clinical relevance. A well-known method for patient selection is the immunohistochemical detection of EGFR expression in biopsy-derived sections of HNSCC (6,7). However, uncertainties such as biopsy accuracy and tumor heterogeneity affect the specificity and reliability of the procedure, and results showed prognostic value but not predictive value (8). In the last decades, molecular imaging has played an important role as a noninvasive technique for patient selection, monitoring and prediction of outcome. EGFR-targeted biomarkers have been widely investigated; cetuximab and its analogues have been labeled with several radionuclides, including $^{111}$In for single photon emission tomography (SPECT) and $^{124}$I, $^{68}$Ga, $^{89}$Zr and $^{86}$Y for positron emission tomography (PET) (9).
While whole antibodies accumulate efficiently in the tumor, it requires 4–7 days before non-target radioactivity has reached sufficiently low levels to enable acquisition of high contrast SPECT images (10). Conversely, F(ab’)2 fragments demonstrate high tumor-to-background signal ratios at earlier time points, while retaining tumor-binding characteristics similar to those of whole antibodies (11,12). Previous in vivo studies have shown that 111In-cetuximab-F(ab’)2 can visualize EGFR accessibility in mice. It displayed a differential uptake in head and neck xenografts, with varying levels of EGFR expression and differential response to treatment with radiotherapy and/or cetuximab (13–15). The aim of this study is to develop a PET-based imaging agent with clinical perspective for imaging EGFR, as PET has a higher resolution and allows more accurate quantitative analyses of images than SPECT (16). The decay properties of 64Cu include an intermediate half-life of 12.7 h, which is equal to the circulatory half-life of F(ab’)2 fragments. Its low positron emission energy (E_{cm} = 0.65 MeV) provides high spatial resolution for PET and autoradiography (17). Therefore, the combination of PET, cetuximab-F(ab’)2, and 64Cu creates certain potential for the clinical imaging of EGFR.

2. MATERIAllS AND METHODS

2.1. Synthesis and radiochemistry

Cetuximab-F(ab’)2 was produced as described previously and purified by gel filtration chromato graphy on a G25M Sephadex column (14). Cetuximab-F(ab’)2 was conjugated with 2,2’-(7-(1-carboxy-4-((4-iodothiocyanatobenzyl)amino)-4-oxobuty)-1,4,7-triazonane-1,4-diyl)diacetic acid (ITC-NODAGA) (CheMatech, Dijon, France) in 0.1 M NaHCO3, pH 9.5, for 3 h at room temperature using a 20-fold molar excess of ITC-NODAGA. Unbound ITC-NODAGA was removed by di-alysis against NH4OAc (0.25 M, pH 5.5).

64Cu (t1/2 = 12.7 h, β+ = 17%, β− = 39%, EC = 44%) in the form of 64CuCl2 was produced via the 64Ni(p, n)64Cu nuclear reaction, as described previously (18,19). 64Cu was formulated as 5 MBq/μL in 0.04 M HCl solution. The antibody fragments (1 μg) were labeled with 64Cu (5 MBq, 1 μL) in 25 μL of 2-((N-morpholinol)ethanesulfonic acid (MES) buffer, pH 5.5 at room temperature. After 30 min, labeling efficiency was determined using instant thin-layer chromatography (ITLC) on TEC Control chromatography strips (Biodex, Shirley, NY, USA), with 0.1 M citrate buffer, pH 6.0, as the mobile phase. Radiochemical purity of 64Cu-cetuximab exceeded 98% in all preparations. The specific activity of the tracer was 500 GBq/μmol.

2.2. In vitro characterization

64Cu-cetuximab-F(ab’)2 (1.2 MBq, 10 μL) was added to 100 μL of murine plasma to assess stability. The mixture was incubated at 37°C. After 4 and 26 h incubation, aliquots of 1 μL were analyzed by ITLC (0.1 M citrate buffer, pH 6.0).

2.3. Tumor models

Human HNSCC UT-SCC-8 or UT-SCC-45 xenografts (1 mm3, 1 × 106 cells) were injected in the right hind leg of male athymic BALB/c nu/nu mice (6–10 weeks). UT-SCC-8 originated from a supraglottic larynx and UT-SCC-45 from a primary cancer of the floor of the mouth (University of Turku). Animals were housed in filter-topped cages in a specific-pathogen-free unit in accordance with institutional guidelines. Experiments started 48 days (UT-SCC-8) or 41 days (UT-SCC-45) after transplantation. Tumor volume was estimated using the formula (4/3)πr2/2. At the start of the experiment, the mean size of the tumors was 764 cm3, with a standard error of 150 cm3. The experiment procedures were reviewed by the Ethics Committee on Animal Experimentation of the University of Turku and approved by the Provincial State Office of Western Finland.

2.4. Biodistribution and PET/CT studies

64Cu-cetuximab-F(ab’)2 (21 ± 2.6 MBq, 15 μg, 250 μL) was intravenously injected in the tail vein followed by 100 μL of 0.9% NaCl to flush the catheter. One additional mouse of each tumor model was injected with an excess dose of unlabeled cetuximab (1 mg) 3 days prior to tracer injection. Saturation of the antigen is often achieved by co-injection; however, as cetuximab F(ab’)2 targets the tumor much faster than cetuximab IgG, injection with unlabeled antibody is administered 3 days before F(ab’)2 tracer injection. Mice were anesthetized with 2.5% isoflurane and positioned in an Inveon PET/CT scanner (Siemens Medical Solutions, Knoxville, TN, USA) for an 8 min CT acquisition and 30 min PET scan in list mode, with an energy window of 350–650 keV. The PET scanner had an axial field of view of 12.7 cm and a spatial resolution of 1.4 mm full width at half-maximum. During scans, the body temperature of the mice was kept at 37°C with a heating pad on the scanner bed. Subsequently, mice were euthanized and the biodistribution of the radiolabel at 24 h p.i. was determined.

2.5. Immunohistochemistry and autoradiography

Frozen tumor sections (5 μm) were cut and mounted on poly-L-lysine-coated slides for autoradiography. Slides were exposed to an imaging plate (BAS-TR2025, Fuji Photo Film, Tokyo, Japan) for three days. The imaging plates were scanned using a Fuji BAS-5000 analyzer at a pixel size of 25 × 25 μm2. Images were processed using a computerized image analysis program (Aida Image Analyzer software 4.19, Raytest Isotopenmessgeräte, Taubrnhardt, Germany).

After autoradiography, the same slides were stained immunohistochemically. Tumor sections were fixed in acetone at 4°C for 10 min. Subsequently, sections were rehydrated in phosphate-buffered saline (PBS) and stained for EGFR. Primary and secondary antibodies were diluted in primary antibody diluent (Abcam, Cambridge, UK). Between all consecutive steps of the staining process, sections were rinsed three times each for 5 min in 0.1 M PBS, pH 7.4 (Klinpath, Duiven, The Netherlands). After rehydration in PBS, sections were incubated with goat anti-EGFR antibody 1:50 (Santa Cruz Biotechnology, Dallas, Texas, USA) and subsequently with donkey anti-goat Cy3, 1:600 (Jackson Immunoresearch, West Grove, PA, USA), after which slides were mounted in Fluorostab (ICN, Basingstoke, UK).

2.6. Image analysis

PET images were reconstructed using an OSEM 3D algorithm of two iterations followed by maximum a posteriori (MAP, 18 iterations) integrative algorithms (Inveon Acquisition Workplace, version 1.5, Siemens Preclinical Solutions).

Reconstructed images were analyzed with Siemens Inveon Research Workplace software (version 4.0, Siemens Preclinical Solutions) by manually placing regions of interest (ROIs) around the tumor. Quantification of tracer uptake in tumor ROIs of the attenuation-corrected slices was obtained by calculating the maximum standardized uptake values (SUVmax) by correcting...
for the injected activity, injection time (decay) and bodyweight. SUV\textsubscript{max} was chosen as it is more reproducible than SUV\textsubscript{mean} since the maximum value within an ROI is typically less dependent on ROI position with respect to small spatial shifts.

Immunohistochemically stained tumor sections were analyzed using a digital image analysis system, as described previously (20). In short, whole-tissue sections were scanned (magnification ×10, Axioskop, Zeiss, Göttingen, Germany) and gray-scale images (pixel size, 2.59 × 2.59 μm\textsuperscript{2}) were obtained for vessels, EGFR and nuclei, and subsequently converted into binary images. Using ImageJ software (version 1.43m, JAVA-based image-processing package), the number of positive pixels for EGFR staining (EGFR\textsuperscript{-}PET) was divided by total tumor area. Thresholds for segmentation of the fluorescent signals were set above the background staining for each marker. Areas of necrosis were excluded from analysis by drawing ROIs.

Colocalization analysis was performed on autoradiography and immunohistochemical gray-value images (grayscale range 0–255). Images were overlaid using Photoshop (CS4, version 11.0.2, San Jose, CA, USA), and the pixel and figure size of the immunohistochemistry images was rescaled to match that of the autoradiography images for alignment (25 × 25 μm\textsuperscript{2}) and was successively upscaled (100 × 100 μm\textsuperscript{2}) to compensate for image co-registration errors and scattering of the tracer signal in the autoradiography images. After alignment, ROIs drawn previously for excluding necrosis in immunohistochemical analysis were masked in autoradiography images. Co-registered pixel gray-values and overlap coefficients were determined with ImageJ using the JACoP plugin package (21). Positioning accuracy between the autoradiography and immunohistochemistry images, as reported by the overlap coefficient calculated with the ImageJ JACoP plugin, was over 95%.

2.7. Statistics

Statistical analyses were performed using Prism software version 6.0 (GraphPad, San Diego, CA, USA). Significance was tested with the non-parametric Mann–Whitney test and/or repeated measures ANOVA. The non-parametric Spearman or parametric Pearson test was used accordingly, and a p-value ≤ 0.05 was considered significant. Data values are represented as mean ± standard deviation.

3. RESULTS

3.1. In vitro characterization

\textsuperscript{64}Cu-cetuximab-F(ab\textsuperscript{2})\textsubscript{2} was moderately stable in murine plasma. After 2 h and 26 h incubation at 37°C, 90% and 84% respectively of the radioactivity was still associated with the intact radiotracer.

3.2. Biodistribution

At 24 h p.i., the tumor biodistribution of \textsuperscript{64}Cu-cetuximab-F(ab\textsuperscript{2})\textsubscript{2} in the UT-SCC-8 and UT-SCC-45 xenografts was similar: 5.48 ± 1.29% ID/g and 4.23 ± 2.06% ID/g, respectively (p = 0.36). Mice that were pre-injected with an excess of unlabeled cetuximab had lower tumor uptake: 0.82% ID/g for UT-SCC-8 and 0.81% ID/g for UT-SCC-45. The biodistribution of \textsuperscript{64}Cu-cetuximab-F(ab\textsuperscript{2})\textsubscript{2} in mice with HNSCC xenografts is summarized in Fig. 1.

24 h p.i., tumor-to-blood ratios in the two models were similar: 12.8 ± 5.2 for UT-SCC-8 and 10.9 ± 6.4 for UT-SCC-45 (p = 0.53). The kidneys and liver were the normal tissues with the highest uptake: 7.9 ± 1.2% ID/g and 8.0 ± 0.7% ID/g for UT-SCC-8; 6.7 ± 3.9% ID/g and 9.0 ± 0.5% ID/g for UT-SCC-45, respectively.

3.3. PET studies

Images showed a higher \textsuperscript{64}Cu-cetuximab-F(ab\textsuperscript{2})\textsubscript{2} uptake in UT-SCC-8 tumors than in UT-SCC-45 tumors (Fig. 2). The SUV\textsubscript{max} differed significantly: 1.5 ± 1.0 for UT-SCC-8 and 0.8 ± 0.2 for UT-SCC-45 (p < 0.05) (Fig. 3). SUV\textsubscript{max} values of the tumors of the mice pre-injected with excess unlabeled cetuximab were clearly lower: SUV\textsubscript{max} was 0.4 for both UT-SCC-8 and UT-SCC-45.

3.4. Immunohistochemistry and autoradiography

UT-SCC-8 had a significantly higher expression of EGFR compared with UT-SCC-45; the receptor-positive fraction was 0.50 ± 0.19 versus 0.12 ± 0.08 (p < 0.005), respectively (Fig. 4). Autoradiography images of \textsuperscript{64}Cu-cetuximab-F(ab\textsuperscript{2})\textsubscript{2} correlated with spatial EGFR expression as determined immunohistochemically for both tumor lines: the correlation coefficient (r) was 0.86 ± 0.06 for UT-SCC-8 and 0.90 ± 0.06 for UT-SCC-45 (Fig. 5). The tumor from one mouse was omitted from the analysis owing to inability to align after skewing of the slide.

4. DISCUSSION

The present study shows that \textsuperscript{64}Cu-cetuximab-F(ab\textsuperscript{2})\textsubscript{2} can be used as a tracer to determine EGFR expression in HNSCCs noninvasively with PET. A significant correlation was found between the intratumoral localization of \textsuperscript{64}Cu-cetuximab-F(ab\textsuperscript{2})\textsubscript{2} as determined by autoradiography and EGFR expression as determined immunohistochemically, indicating that the EGFR expressed on the tumor cells is specifically targeted by the tracer.

Accumulation of \textsuperscript{64}Cu-cetuximab-F(ab\textsuperscript{2})\textsubscript{2} in the tumor was EGFR mediated in both UT-SCC-8 and UT-SCC-45, as it could be inhibited by pre-injection of an excess of unlabeled cetuximab. Tumor uptake of \textsuperscript{64}Cu-cetuximab-F(ab\textsuperscript{2})\textsubscript{2} in terms of % ID/g was similar to that of the \textsuperscript{111}In-labeled cetuximab-F(ab\textsuperscript{2})\textsubscript{2} tracer as described previously (13–15). Cetuximab has been radiolabeled with \textsuperscript{64}Cu in several other studies, using DOTA as a chelator (22–27). However, this resulted in increased radioactivity levels...
in the liver, which was attributed to transchelation of Cu$^{2+}$ from DOTA to superoxide dismutase and ceruloplasmin (28). Previous reports have demonstrated the enhanced thermodynamic stability of Cu-triazamacrocyclic complexes compared with Cu-DOTA derivatives (29–35).

Yaromina et al. determined the TCD$_{50}$ of these UT-SCC tumors, i.e. the dose necessary to locally control 50% of the tumors, and showed that the radiosensitivities of UT-SCC-8 and UT-SCC-45 were similar, with TCD$_{50}$ values of 53 Gy and 45 Gy, respectively (36). PET images and SUV$_{max}$ quantification showed a significant difference in $^{64}$Cu-cetuximab-F(ab$′$)$_2$ uptake between the two HNSCC tumors, which might reflect the sensitivity of these tumors towards EGFR inhibitors. The $^{64}$Cu-cetuximab-F(ab$′$)$_2$ tumor uptake of UT-SCC-45 was lower, which suggests that this tumor might not benefit from EGFR-targeted therapy, while the tumor with higher tracer uptake, UT-SCC-8, might be more sensitive to EGFR inhibition. However, more HNSCC tumor models should be analyzed to validate whether tracer uptake correlates with sensitivity to cetuximab treatment.

Some discrepancy was noted between PET-derived SUV$_{max}$ and ex vivo biodistribution data, the latter showing no significant difference of tracer uptake in the two HNSCC tumors. The lack of a significant difference in tumor uptake could be due to necrotic areas in both UT-SCC tumor lines, thereby increasing the apparent tumor weight and distorting the uptake per gram tumor tissue, but having no effect on the SUV$_{max}$ as necrotic areas with low uptake are excluded from that analysis.

In a previous study, $^{111}$In-cetuximab-F(ab$′$)$_2$ has been shown to be able to measure radiation-induced changes of EGFR expression. Irradiated SCCNij202 tumors exhibited an increase in $^{111}$In-cetuximab-F(ab$′$)$_2$ uptake up to 14 days after treatment, which correlated with an increase of available membranous EGFR as determined immunohistochemically (15). Intratumoral localization of EGFR in HNSCCs is heterogeneous, and to allow accurate tracer quantification and reduction of partial volume effects it is necessary to acquire high-resolution images. Clinical PET images have a better spatial resolution than SPECT images (2–5 mm versus 7–10 mm), encouraging the development of PET tracers for this application (16). The ability of $^{64}$Cu-cetuximab-F(ab$′$)$_2$ to monitor treatment has not yet been established, but the current study shows that this PET tracer is able to accurately measure heterogeneous EGFR expression in tumors within a relatively short time period.

Optimization of kinetics and specificity of PET tracers is necessary in order to advance in a clinical setting. Longitudinal
measurement of EGFR expression could elucidate patient-specific tumor characteristics during the course of the disease and during treatment, thereby facilitating personalized treatment. The preclinical data described here suggest the potential of $^{64}$Cu-cetuximab-F(ab$'$$)_{2}$ as a clinical EGFR-targeting tracer.

5. CONCLUSION

This report described the successful NODAGA conjugation and $^{64}$Cu labeling of cetuximab-F(ab$'$$)_{2}$ with high specific radioactivity. PET studies showed the potential of the $^{64}$Cu-cetuximab-F(ab$'$$)_{2}$ radiotracer to determine systemically targetable EGFR expression in heterogeneous tumors with superior image quality at a relatively early time interval. The tracer accumulated rapidly in two different HNSCC tumor models with good tumor-to-background signal at 24 h after injection. Tracer uptake in the tumor models studied correlated to EGFR expression as measured by immunohistochemistry. Imaging with the PET tracer $^{64}$Cu-cetuximab-F(ab$'$$)_{2}$ shows clinical promise in determining intratumoral EGFR distribution, and might predict cetuximab accumulation and subsequent response to EGFR-inhibitor treatment.

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REFERENCES


11. Heskamp S, van Laarhoven HW, Molkenboer-Kuenen JD, Bouwmeester WH, van der Graaf WT, Oyen WJ, Boerman OC. Optimization of IGF-1R SPECT/CT imaging using 111In-labeled Fab′(2) and Fab fragments of the monoclonal antibody R1507. Mol Pharm 2012; 9(8): 3022–3031.


