Cetuximab-F(ab')$_2$-SPECT and FDG-PET for prediction and response monitoring of combined modality treatment of human head and neck carcinomas in a mouse model

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ABSTRACT

Treatment of head and neck squamous cell carcinomas (HNSCC) with radiotherapy and EGFR-inhibitor cetuximab shows an improved response in a subgroup of patients. The aim of this study was to non-invasively monitor treatment response by visualizing systemically accessible EGFR with $^{111}$In-cetuximab-F(ab')$_2$ while simultaneously evaluating tumor metabolism with $^{18}$F-FDG PET during combined modality treatment.

Methods: Eighty mice with patient-derived HNSCC xenografts, SCCNij202 or SCCNij185, were imaged with SPECT/CT using $^{111}$In-cetuximab-F(ab')$_2$ (5 µg, 28 ± 6.1 MBq, 24 h p.i.) followed by PET imaging with $^{18}$F-FDG (9.4 ± 2.9 MBq, 1 h p.i.). Scans were acquired 10 days prior to treatment with either single-dose 10 Gy, cetuximab alone, cetuximab + 10 Gy combined or untreated controls. Scans were repeated 18 days post treatment. Tumor growth was monitored up to 120 days after treatment. EGFR expression was evaluated immunohistochemically.

Results: SCCNij202 responded to combined treatment ($p<0.01$) and cetuximab treatment alone ($p<0.05$), but not to irradiation alone ($p=0.13$). SCCNij185 responded to combined treatment ($p<0.05$) and irradiation ($p<0.05$), but not to cetuximab treatment alone ($p=0.34$). $^{111}$In-cetuximab-F(ab')$_2$ uptake (tumor-to-liver ratio, scan2-scan1) predicted response to therapy. A positive response to treatment significantly correlated with a reduced tracer uptake in the tumor in the second SPECT scan compared to the first scan ($p<0.005$, $p<0.05$ for SCCNij202 and SCCNij185, respectively). Resistance to therapy was characterized by a significantly increased $^{111}$In-cetuximab-F(ab')$_2$ tumor uptake; tumor-to-liver ratio was 2.2 ± 0.6 to 3.5 ± 1.2, $p<0.01$ for (irradiated) SCCNij202 and 1.4 ± 0.4 to 2.0 ± 0.3, $p<0.05$ for (cetuximab-treated) SCCNij185, respectively. FDG-PET tumor uptake (SUVmax, scan2-scan1) correlated with tumor response for
SCCNij202 (p<0.01) but not for SCCNij185 (p=0.66). EGFR fractions (fEGFR) were significantly different: 0.9 ± 0.1 (SCCNij202) and 0.5 ± 0.1 (SCCNij185) (p<0.001). fEGFR was significantly lower for irradiated SCCNij202 tumors, compared to controls (p<0.005).

**Conclusion:** $^{111}$In-cetuximab-F(ab')$_2$ predicted and monitored the effects of EGFR-inhibition and/or irradiation during treatment in both head and neck carcinoma models investigated, whereas $^{18}$F-FDG-PET only correlated with tumor response in the SCCNij202 model. This emphasizes the additional value of the $^{111}$In-cetuximab-F(ab')$_2$ tracer and can aid in evaluating future treatments with EGFR-targeted therapies.

Keywords: SPECT, PET, radiotherapy, cetuximab, HNSCC
INTRODUCTION

Squamous cell carcinoma of the head and neck is the sixth leading cause of cancer death worldwide (1). Risk factors include alcohol and tobacco use and oral infection by HPV (2). Treatment of advanced disease primarily consists of concurrent chemotherapy and radiotherapy. This combined treatment has improved outcome, albeit only modestly, but also increases treatment-related morbidity (3,4). Cetuximab, an epidermal growth factor receptor (EGFR) inhibitor, has been shown to significantly improve overall survival and locoregional control through radiosensitization with limited long-term morbidity (5,6). However, only a fraction of the patients will benefit from the addition of cetuximab treatment and before treatment it is unclear which patients are most likely to benefit. Patient stratification is pivotal to improve clinical response rates and biomarker development for prediction or response to treatment is widely investigated.

A proposed biomarker in HNSCC is tumor EGFR expression as the EGFR is significantly overexpressed compared to normal epithelial tissues (7). However, in a phase II study by Wierzbicki et al it was shown that even without detectable EGFR, cetuximab as a monotherapy for colorectal carcinomas could be clinically effective and recently, an inverse relationship between EGFR expression and response was noted by Hartmann et al (8,9). Nuclear medicine imaging advances on biopsy/single cell-dependent techniques as it allows non-invasive assessment of the EGFR in the intact tumor, accounting for intratumoral variables like vasculature, blood supply and interstitial fluid pressure. Additionally, imaging can be repeated during treatment and serve as a monitoring tool.

Several PET markers like $^{18}$F-FDG, $^{18}$F-FLT and $^{18}$F-FMISO have been investigated in HNSCC patients and have shown to be of prognostic value (10-12), but do not aid in treatment selection. In previous studies, an anti-EGFR tracer, $^{111}$In-cetuximab-F(ab')2, was developed and evaluated in several preclinical setups. It has been shown to
visualize systemically accessible EGFR in multiple HNSCC models as well as the change in accessible EGFR in response to single-dose irradiation (13,14). Here, we assess its potential relevance in two HNSCC xenograft models and include three treatment arms of irradiation, cetuximab and the combination therapy to evaluate its predictive value and its role in response monitoring.
MATERIALS AND METHODS

Tumor Models
Six to ten weeks old athymic BALB/c nu/nu mice were xenografted subcutaneously (s.c.) in the right hind leg with viable tumor tissue (ca. 1 cm$^3$, approx.1 x10$^6$ tumor cells) of the serial passaged human HNSCC lines SCCNij202 or SCCNij185. Animals were housed in filter-topped cages in accordance with institutional guidelines. Experiments started 3 weeks (SCCNij202) or 8 weeks (SCCNij185) after transplantation. The Animal Welfare Committee of the Radboud University Medical Center approved the animal experiments.

Experimental Set-up
For each tumor model, 60 animals (total n = 120) were assigned into four treatment groups; radiotherapy, cetuximab, cetuximab combined with radiotherapy and control (n=15 per group). Tumor volume was estimated using the formula; 4/3 π r$_1$ x r$_2$ x r$_3$. For growth delay, tumors were measured twice a week and the endpoint was reached when tumor volume doubled (SCCNij185) or tripled (SCCNij202) in size compared to start volume. Different endpoints were chosen for both tumor models as SCCNij202 had a shorter doubling time than SCCNij185. Maximum follow-up time was 120 days. Non-responders were defined as those reaching tumor doubling or tripling of start volume. Reduction in tumor size or growth delay (below doubling or tripling volume) was considered a response of the tumor to therapy.

Baseline $^{111}$In-cetuximab-F(ab’)$_2$ SPECT/CT and $^{18}$F-FDG PET scans of 80 mice were acquired 10 days before treatment. Radiotherapy consisted of a single dose of 10Gy X-rays on the tumor-bearing right hind leg (320 kV, dose rate 3.8 Gy/min, X-RAD, RPS Services Limited). Mice received a therapeutic dose of 1.0 mg cetuximab by i.p. injection, 3 days prior to radiotherapy. 18 days after treatment, all mice were subjected
to a second SPECT and PET scan and followed in a growth delay set up. From the remaining 40 mice, five (SCCNij202) or four (SCCNij185) of each group were euthanized for immunohistological evaluation 18 days after treatment.

SPECT and PET Imaging

$^{111}$In-cetuximab-F(\text{ab}')$_2$ (specific activity of 400 GBq/µmol, radiochemical purity > 95%) was produced as described previously (14). SPECT images were acquired 24 h after i.v. injection with $^{111}$In-cetuximab-F(\text{ab}')$_2$ (5 µg, 28 ± 6.1 MBq, 200 µl per mouse) using an ultra high-resolution animal SPECT/CT scanner (USPECT-II; MILabs). Mice were scanned in prone position under general anesthesia (isoflurane/compressed air) using the 1.0-mm diameter multi-pinhole collimator tube. SPECT scans were acquired for 60 min, followed by a 180 second CT scan. Subsequently, mice were injected with $^{18}$F-FDG (9.4 ± 2.9 MBq) (GE) and kept under general anesthesia for one hour before starting 20 min PET imaging with an Inveon small-animal PET scanner (Siemens Preclinical Solutions) followed by a 400-second transmission scan using the built-in $^{57}$Co source (energy window 120-125 keV) for attenuation correction.

Excised tumors were snap-frozen in liquid nitrogen for immunohistochemical staining purposes. Due to technical issues with the scanners, not all mice could be scanned at the second time point.

Immunohistochemistry

Frozen tumor sections (5 µm) were cut and mounted on poly-L-lysine–coated slides for immunohistochemistry. First, tumor sections were fixed in acetone in 4°C for 10 min. Subsequently, slides were washed and stained for EGFR, vessels and nuclei. Primary and secondary antibodies were diluted in primary antibody diluent (PAD, Abcam).
Between all consecutive steps of the staining process, sections were rinsed three times for 5 min in 0.1 M PBS, pH 7.4 (Klinipath).

After rehydration in PBS, sections were incubated with goat anti-EGFR antibody 1:50 (Santa Cruz Biotechnology Inc) and subsequently with donkey anti-goat Cy3, 1:600 (Jackson Immunoresearch). To stain the blood vessels, all sections were incubated with undiluted 9F1 supernatant (anti-mouse endothelium) (15), for 45 min at 37°C followed by incubation with chicken anti-rat-Alexa647, 1:100 (Invitrogen Molecular Probes). All nuclei were stained with 0.5 µg/ml Hoechst 33342 (Sigma) after which slides were mounted in Fluorostab (ICN Biomedicals). One adjacent section per tumor was Hematoxylin and Eosin (HE) stained to help distinguish necrotic areas and non-tumor tissue from viable tumor areas.

**Image Analysis**

SPECT scans were reconstructed with MI Labs reconstruction software, using an ordered-expectation maximization algorithm with a voxel size of 0.375 mm. Tumor-to-liver pixel value ratios (T/L) were determined by drawing regions of interest (ROIs) around the tumor (thresholded at 40% of the maximum signal) and within the liver (Inveon Research Workplace software version 4.0, Siemens Preclinical Solutions). The relative difference between T/L ratios from the first and second scan are represented as (scan2-scan1)/scan1.

PET images were reconstructed using an OSEM 3D algorithm of two iterations followed by maximum a posteriori (MAP, 18 iterations, $\beta = 0.05$) reconstruction optimized for uniform resolution (Inveon Acquisition Workplace, version 1.5, Siemens Preclinical Solutions). PET images were analyzed using Siemens Inveon Research Workplace software (version 4.0; Siemens Preclinical Solutions). ROIs were manually drawn around the tumor. Tumors were thresholded at 50% of the maximum signal. 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 and bodyweight. The relative difference between the SUVmax from the first and second scan are represented as (scan2-scan1)/scan1.

After immunohistochemical staining, tumor sections were analyzed using a digital image analysis system, as described previously (16). In short, whole-tissue sections were scanned (magnification x 10, Axioskop, Zeiss) and gray-scale images (pixel size, 2.59 × 2.59 μm) were obtained for EGFR, vessels and nuclei and subsequently converted into binary images. Using ImageJ software (version 1.43m, JAVA-based image-processing package), the amount of positive pixels for EGFR staining was divided by total tumor area, providing the fraction of EGFR (fEGFR). Mean intensity of the EGFR staining (iEGFR) was determined by dividing EGFR pixel grey-values (range 0 - 4095, 12 bits) by positive EGFR staining 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.

**Statistics**

Statistical analyses were performed using Prism software version 6.0e (Graphpad). Significance of tumor response was tested using Kaplan-Meier survival curves and Cox proportional-hazards regression. The nonparametric Spearman or parametric Pearson test was used accordingly and a p-value ≤ 0.05 was considered significant. Data are represented as mean ± standard deviation (SD).
RESULTS

Growth Delay
Average tumor volume at onset of treatment was 228 ± 101 mm³ and 132 ± 78 mm³ for SCCNij202 and SCCNij185 respectively, and did not differ between treatment groups. SCCNij202 responded to combined (p<0.01) and cetuximab treatment alone (p<0.05), but not to irradiation (p=0.13) (Fig. 1A). SCCNij185 responded to combined treatment (p<0.05) and irradiation (p<0.05), but not to cetuximab treatment alone (p=0.34) (Fig. 1B).

\[^{111}\text{In}-\text{cetuximab-}F(ab')_2\text{ SPECT}\]
\[^{111}\text{In}-\text{cetuximab-}F(ab')_2\text{ tumor uptake decreased significantly in SCCNij202 and SCCNij185 tumors when treated with combined modality treatment of cetuximab and single-dose 10 Gy irradiation within 2.5 weeks after treatment (p<0.001 and P<0.01, respectively) (Fig 2, 3). Resistance to therapy, i.e. lack of growth delay or increase in tumor volume up to 120 days, was characterized by a significantly increased }^{111}\text{In-cetuximab-}F(ab')_2\text{ tumor uptake compared to the pre-therapy scan, 18 days after treatment. The tumor-to-liver ratio increased from 2.2 ± 0.6 to 3.5 ± 1.2, p<0.01 for (irradiated) SCCNij202 and from 1.4 ± 0.4 to 2.0 ± 0.3, p<0.05 for (cetuximab-treated) SCCNij185, respectively. Tumors that responded to therapy (those not reaching volume doubling or tripling, respectively) had a significantly reduced tracer uptake in the tumor in the second SPECT scan compared to the first scan (p<0.005 and p<0.05 for SCCNij202 and 185, respectively) (Fig 4A, B).\

\[^{18}\text{F-FDG PET}\]
FDG-PET tumor uptake correlated with tumor response for SCCNij202 (p<0.005), but not for SCCNij185 (p=0.66) (Fig 4C, D). The decrease of $^{18}$F-FDG uptake in the tumor was significant for SCCNij202 tumors treated with cetuximab and with the combination therapy; 1.59 ± 0.36 to 0.70 ± 0.34, p<0.005 and 1.43 ± 0.18 to 0.74 ± 0.21, p<0.0001, respectively (Fig 2, 5).

**Immunohistochemistry**

EGFR fractions of SCCNij202 and SCCNij185 control tumors obtained by quantification of EGFR by immunohistochemistry, were significantly different: 0.9 ± 0.1 (SCCNij202) and 0.5 ± 0.1 (SCCNij185) (p<0.05). fEGFR was significantly lower for irradiated SCCNij202 tumors, compared to controls (p<0.005) (Fig 6A). The intensity of the EGFR staining was significantly elevated for irradiated SCCNij202 tumors (p<0.05) (Fig 6B). SCCNij185 did not show any significant differences between treated and control groups for fEGFR or iEGFR.
DISCUSSION

Stratification of patients could lead to an improved approach to treating heterogeneous diseases like head and neck cancer. Here we show that \(^{111}\text{In-cetuximab-F(ab')}_2\) can be an imaging biomarker for monitoring combined modality treatment and predicts outcome to therapy in two head and neck tumor models.

The effectiveness of combining EGFR-inhibitor cetuximab with radiotherapy has been established in the Bonner trial (5). The mechanism underlying this effect of cetuximab has been extensively studied and is in part mediated by inhibition of DNA damage repair after radiation-induced damage and by promoting apoptosis of the tumor cells (17-20). In our study, SCCNij202 and SCCNij185 tumors both respond to the combination therapy of cetuximab and irradiation. Treatment efficacy seemed to be facilitated by a greater degree through EGFR-inhibition for SCCNij202 as seen by the percentage of tumor response when given cetuximab alone. For SCCNij202, which has an increased EGFR gene copy number, it emphasizes the addiction to, and dependency on, EGFR-ligands for continued proliferation and survival (21,22). A significant increase in \(^{111}\text{In-cetuximab-F(ab')}_2\) uptake in the tumor 2.5 weeks after treatment was seen in tumors resistant to therapy. For radiation-resistant SCCNij202, we found an increase of tracer uptake in irradiated tumors along with an increase in iEGFR, which points to an increase in membrane receptor availability, as described previously (23). This may represent a compensation mechanism where the increase in systemically available EGFR enhances EGFR-signaling promoting cell survival, especially in a heavily EGFR-reliant tumor model such as SCCNij202. For the SCCNij202 cetuximab treated group, showing a good response to treatment, we observed no significant change in tumor fEGFR, iEGFR or \(^{111}\text{In-cetuximab-F(ab')}_2\) tracer uptake after treatment. Cetuximab therapy is known to induce internalization and degradation of the EGFR (24), but it has been suggested that
the effectiveness of EGFR-inhibitors depends more on inhibition of the AKT and ERK downstream signaling rather than membranous downregulation of the EGFR (25). This mechanism would be in line with our findings in the cetuximab/combined therapy treated SCCNij202 tumors, where the expression of EGFR and tumor tracer uptake did not change.

SCCNij185 tumors showed a more classical response to therapy; cetuximab enhancing irradiation induced damage to tumor cells, as SCCNij185 tumors were responsive to irradiation, but not to cetuximab treatment alone. Resistance to cetuximab was characterized by a significant increase in \(^{111}\)In-cetuximab-F(ab')\(_2\) tumor uptake 18 days after treatment, though no differences in iEGFR or fEGFR were found, thereby differentiating from the mechanism proposed for SCCNij202. This supports the current understanding that EGFR expression levels, as determined immunohistochemically, cannot predict therapy response (26-28). Intracellular signaling routes can have a pivotal role independent of high EGFR expression on tumors. In several studies, the persistent activation of the MAPK and/or P13-K/AKT pathway has been shown to play an important role in EGFR-inhibitor resistance (25,29,30). SCCNij185 is known to express high endogenous levels of phosphorylated AKT, which might explain the insensitivity towards cetuximab treatment in this model (21).

As both SCCNij202 and SCCNij185 tumors respond to combination therapy but respond differently to single treatment, initial \(^{111}\)In-cetuximab-F(ab')\(_2\) scans prior to treatment cannot be used as a marker for selection of EGFR-inhibitor treatment. However, within 18 days after treatment, the difference in \(^{111}\)In-cetuximab-F(ab')\(_2\) uptake could predict tumor response. This enables the ability to monitor therapy response, and thus to modify the therapeutic regimen. Especially relevant is the increase of tracer uptake in the tumor as it correlates with non-responders, i.e. lack of growth delay or increase in tumor volume. More factors are necessary to predict individual response, as evidenced by the
fact that some tumors show a decrease in tumor tracer uptake in the second scan, but do not show a decrease in tumor size after therapy, and vice versa. Interfering factors could include the amount of tumor necrosis, presence of edema or the variation in tumor size before start of treatment.

In a previous study, we assessed the response to irradiation with $^{111}$In-cetuximab-F(\(ab')_2\) imaging in SCCNij202 and SCCNij167 (23). As these two models portray clinical extremes in cetuximab response and high EGFR (SCCNij202) versus low EGFR (SCCNij167), it was deemed of added value to investigate a model with moderate EGFR expression and cetuximab non-response. In combination with the additional treatment regimens, which provides a more in-depth evaluation, this study elucidated the value of cetuximab-F(\(ab')_2\) imaging as it showed an increased tracer uptake in non-responding tumors to different treatments in both SCCNij tumors.

In patients, response of HNSCC to (chemo)radiotherapy with imaging markers has been evaluated in several clinical studies (31). $^{18}$F-FDG has been shown to have prognostic value, though the search for a predictive or early assessment potential has revealed conflicting results (32,33). In this study, a reduction in $^{18}$F-FDG SUVmax correlated with tumor response and served as a predictive marker in SCCNij202 tumors, consistent with our previous findings (23). However, no difference in uptake was seen in SCCNij185 tumors, regardless of the applied treatment. This emphasizes the need to apply tumor-specific tracers and warrants further investigation of additional early response and predictive markers for combined modality treatment of radiotherapy with EGFR-inhibitors.

**CONCLUSION**
In this study, we have shown that $^{111}$In-cetuximab-F(ab$'$)$_2$ can be used to predict and monitor the effects of combined modality treatment of EGFR-inhibition and/or irradiation in two head and neck carcinoma models. Whereas $^{18}$F-FDG-PET only correlated to tumor response in SCCNij202, the change in $^{111}$In-cetuximab-F(ab$'$)$_2$ uptake early after treatment correlated to treatment outcome in both SCCNij202 and SCCNij185. Most evident is the increased uptake in non-responding tumors in the two tumor models, allowing visualization of tumor-specific ineffective therapies, hence facilitating early alterations in treatment regimen.

DISCLOSURE

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FIGURE 1. Kaplan-Meijer survival curves showing the response to cetuximab and/or irradiation in SCCNij202 (A) and SCCNij185 (B). Animals were treated with single irradiation dose of 10 Gy, single i.p. injection of 1mg cetuximab, a combination of both or served as controls. n =10 per treatment group per HNSCC tumor type.
FIGURE 2. $^{111}$In-cetuximab-F(ab$'$)$_2$ (dorsal view, 24 h p.i.) and $^{18}$F-FDG (transversal view, 1 h p.i.) uptake. Upper panel: SCCNij202. Lower panel: SCCNij185. Per treatment group, baseline example (left, 10 d pre-treatment) and treated sample (right, 18 d post-treatment) are shown. Tumors are located on the right hind leg (red arrow). Background uptake was present in SPECT images (liver, kidneys, bladder) and PET (bladder).
FIGURE 3. $^{111}$In-cetuximab-F(ab')$_2$ tumor uptake shown as SPECT tumor-to-liver (T/L) ratio for SCCNij202 (A) and SCCNij185 (B). Mice were imaged 10 days before (black bars) and 18 days after (white bars) treatment with cetuximab and/or irradiation. n = 10 per treatment group per HNSCC tumor type. * p < 0.05, ** p < 0.01, *** p < 0.001.
FIGURE 4. The relative tumor-to-liver (T/L) ratio of two $^{111}$In-cetuximab-F(ab')$_2$ scans (A,B) and the relative SUVmax ratio of two $^{18}$F-FDG scans (C,D) dichotomized in responders and non-responders. A positive response to treatment (left column) significantly correlated with a reduced cetuximab-F(ab')$_2$ tracer uptake for SCCNij202 (A) and SCCNij185 (B). A positive response to treatment significantly correlated with a reduced FDG tracer uptake for SCCNij202 (C) but not for SCCNij185 (D). Horizontal lines represent grand mean. t 0 = 10 d pre-treatment, t 1 = 18 d post-treatment. ** p < 0.005, * p < 0.05.
FIGURE 5. $^{18}$F-FDG tumor uptake (SUVmax) for SCCNij202 (A) and SCCNij185 (B). Mice were imaged 10 days before (black bars) and 18 days after (white bars) treatment with cetuximab and/or irradiation. $n = 10$ per treatment group per HNSCC tumor type. ** $p < 0.01$, *** $p < 0.001$. 
FIGURE 6.

The immunohistochemical fraction of EGFR (fEGFR) (A) and immunohistochemical intensity of EGFR (iEGFR) (B) for SCCNij202 and SCCNij185 tumors treated with cetuximab and/or irradiation. A significant decrease for fEGFR ($p < 0.001$) and increase for iEGFR ($p < 0.05$) was seen in the irradiated SCCNij202 tumors. $n = 3 - 10$ per treatment group per HNSCC tumor type.