Cancer Therapy: Preclinical

Cetuximab-Based Immunotherapy and Radioimmunotherapy of Head and Neck Squamous Cell Carcinoma

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Abstract

Purpose: To show the relationship between antibody delivery and therapeutic efficacy in head and neck cancers, in this study we evaluated the pharmacokinetics and pharmacodynamics of epidermal growth factor receptor (EGFR)–targeted immunotherapy and radioimmunotherapy by quantitative positron emission tomography (PET) imaging.

Experimental Design: EGFR expression on UM-SCC-22B and SCC1 human head and neck squamous cell cancer (HNSCC) cells were determined by flow cytometry and immunostaining. Tumor delivery and distribution of cetuximab in tumor-bearing nude mice were evaluated with small animal PET using 64Cu-DOTA-cetuximab. The in vitro toxicity of cetuximab to HNSCC cells was evaluated by MTT assay. The tumor-bearing mice were then treated with four doses of cetuximab at 10 mg/kg per dose, and tumor growth was evaluated by caliper measurement. FDG PET was done after the third dose of antibody administration to evaluate tumor response. Apoptosis and tumor cell proliferation after cetuximab treatment were analyzed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling and Ki-67 staining. Radioimmunotherapy was done with 90Y-DOTA-cetuximab.

Results: EGFR expression on UM-SCC-22B cells is lower than that on SCC1 cells. However, the UM-SCC-22B tumors showed much higher 64Cu-DOTA-cetuximab accumulation than the SCC1 tumors. Cetuximab-induced apoptosis in SCC1 tumors and tumor growth was significantly inhibited, whereas an agonistic effect of cetuximab on UM-SCC-22B tumor growth was observed. After cetuximab treatment, the SCC1 tumors showed decreased FDG uptake, and the UM-SCC-22B tumors had increased FDG uptake. UM-SCC-22B tumors are more responsive to 90Y-DOTA-cetuximab treatment than SCC1 tumors, partially due to the high tumor accumulation of the injected antibody.

Conclusion: Cetuximab has an agonistic effect on the growth of UM-SCC-22B tumors, indicating that tumor response to cetuximab treatment is not necessarily related to EGFR expression and antibody delivery efficiency, as determined by PET imaging. Although PET imaging with antibodies as tracers has limited function in patient screening, it can provide guidance for targeted therapy using antibodies as delivery vehicles. Clin Cancer Res; 16(7); 2095–105. ©2010 AACR.
thus an urgent need for both early detection of HNSCC and development of new therapeutic regimens and drugs. Accumulating evidence suggests that targeted biological therapies selectively interfering with cancer cell and/or endothelial cell growth signaling may improve HNSCC patient survival by enhancing radiation and chemotherapy efficacy without increasing treatment-related toxicity (6, 7).

Currently, epidermal growth factor receptor (EGFR) is a valid target for the treatment of cancer patients (8). As a member of the structurally related erbB family of receptor tyrosine kinases (9), EGFR promotes tumor progression in several solid cancers (10). Dysregulation of EGFR is associated with several key features of cancer, such as autonomous cell growth, inhibition of apoptosis, angiogenic potential, invasion, and metastasis (11, 12). Elevated EGFR level has been reported in >95% of HNSCCs compared with normal mucosa (13). In addition, elevated EGFR expression is an independent indicator of poor prognosis and lower survivals in HNSCC patients (14).

EGFR-targeted therapies include monoclonal antibodies, such as cetuximab (IMC-C225, Erbitux) and panitumumab (ABX-EGF, Vectibix), that block the extracellular ligand-binding domain of the receptor and tyrosine kinase inhibitors that prevent the activation of the cytoplasmic kinase portion (15–17). These targeting approaches have shown great promise in preclinical studies (18, 19). It has been reported that radiation activates EGFR signaling, leading to radio resistance by inducing cell proliferation and enhanced DNA repair (20). In patients with locoregionally advanced HNSCC, the combination of cetuximab and high-dose radiation was found to yield superior survival than radiation alone (6). Similarly, the addition of cetuximab to chemotherapy in a large randomized study (21) resulted in significantly longer median survival when compared with chemotherapy alone in patients with recurrent or metastatic HNSCC.

Although clinical results for EGFR targeting with specific antibodies are promising, most studies indicate that only a subgroup of patients receiving the monoclonal antibodies benefit from the drug (22, 23). No correlation has been found between the efficacy of cetuximab and EGFR tumoral staining intensity by immunohistochemistry (24, 25). In addition, cetuximab response has been observed in patients with EGFR-negative tumors (26). Although it has been reported that EGFR gene copy number may predict response to cetuximab, there are concerns regarding reproducibility of such assays (27). Using 64Cu-labeled panitumumab, we have done small animal positron emission tomography (PET) in several HNSCC tumor models (28). Although no correlation with tumor EGFR expression was observed, quantitative PET imaging with radiolabeled antibody enabled visualization and quantification of a number of parameters, including tumor-specific binding, perfusion, vascularity, vascular permeability, and plasma half-life. It provided more comprehensive information about antibody delivery than immunohistochemistry or fluorescence in situ hybridization (29). To explore the potential of immuno-PET to assess EGFR-targeted therapy, we next investigated the therapeutic efficacy of cetuximab and 90Y-cetuximab in two HNSCC tumor models. Our data showed that with very high local accumulation of antibody, UM-SCC-22B tumors are resistant to cetuximab and sensitive to 90Y-cetuximab.

Materials and Methods

**Chemicals and reagents.** 1,4,7,10-Tetraazadodecane-11,11′,14,14′-tetraacetic acid (DOTA) was purchased from Macrocyclics, Inc. FITC and Chelex 100 resin (50–100 mesh) were purchased from Sigma-Aldrich. Water and all buffers were passed through Chelex 100 columns (1 × 15 cm) before use in radiolabeling procedures to ensure that the aqueous buffers were free of heavy metals. PD-10 desalting columns were purchased from GE Healthcare. 64Cu was provided by the University of Wisconsin-Madison. 90Y was obtained from Perkin-Elmer. Cetuximab was purchased from ImClone Systems, Inc.

**Cell lines and tumor models.** The human head and neck squamous carcinoma cell lines SCC1 and UM-SCC-22B were obtained from the University of Michigan. They were maintained in DMEM supplemented with 10% fetal bovine serum, 1% glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin (Invitrogen). All animal experiments were done under a protocol approved by the Stanford University Administrative Panel on Laboratory Animal Care. Subcutaneous HNSCC tumor models were established in 4-wk-old to 6-wk-old female athymic nude mice obtained from Harlan. Typically, 5 × 10⁶ cells suspended in 50 μL of PBS were injected, and the mice were subjected to therapeutic or small animal PET studies when
the tumor volume reached 100 to 200 mm$^3$ (2–3 wk after inoculation).

**Flow cytometry.** HNSCC cells were harvested and washed with PBS containing 0.5% bovine serum albumin. After blocking by 2% bovine serum albumin in PBS, the cells were incubated with cetuximab (10 μg/mL in PBS containing 2% bovine serum albumin). FITC-conjugated donkey anti-human IgG (1:200) was then added and allowed to incubate for 1 h at room temperature. After washing, the cells were analyzed using an LSR flow cytometer (Beckman Coulter). The FITC signal intensity was analyzed using Cell-Quest software (version 3.3, Becton Dickinson).

**MTT assay.** The toxicity of cetuximab to SCC1 and UM-SCC-22B cells was determined by MTT assay. All studies were done with triplicate samples and repeated at least thrice. Briefly, cells were harvested by trypsinization, resuspended in DMEM, and plated in a 96-well plate at 4,000 or 2,000 cells per well. At 72 or 120 h after treatment with different doses of cetuximab (ranging from 0.1 nmol/L to 1.0 μg/mL sterile filtered MTT (Sigma) were added to each well. The unreacted dye was removed after 4 h, and the insoluble formazan crystals were dissolved in 150 μL of DMSO. The absorbance at 570 nm (reference wavelength, 630 nm) was measured with a Tecan microplate reader (Tecan).

**Antibody labeling.** Detailed procedures for FITC and DOTA conjugation of cetuximab have been reported earlier (28). For $^{64}$Cu labeling, $^{64}$CuCl$_2$ (74 MBq) was diluted in 300 μL of 0.1 mol/L sodium acetate buffer (pH 6.5) and added to 50 μg of DOTA-cetuximab. The reaction mixture was incubated for 1 h at 40°C with constant shaking. $^{64}$Cu-DOTA-cetuximab was then purified by PD-10 column using PBS as the mobile phase. The labeling yield was calculated by dividing the radioactivity of $^{64}$Cu-DOTA-cetuximab by the total input radioactivity.

For radioimmunotherapy, $^{90}$Y was added to the DOTA-cetuximab conjugates at 0.68 μg of DOTA-cetuximab per MBq of $^{90}$Y. The reaction mixture was incubated for 1 h at 40°C with constant shaking. The $^{90}$Y-DOTA-cetuximab conjugate was purified by PD-10 column using PBS as the eluent. The radioactive fractions containing $^{90}$Y-DOTA-cetuximab were collected and passed through a 0.2-μm syringe filter for further in vivo experiments.

**Small animal PET and image analysis.** PET imaging of tumor-bearing mice was done on a microPET R4 rodent model scanner (Siemens Medical Solutions) as described earlier (28). The mice were i.v. injected with $^{64}$Cu-DOTA-cetuximab or $^{64}$Cu-DOTA-IgG (Jackson ImmunoResearch Laboratories; 7–8 MBq/6 μg/mouse) and static scans were acquired at 4, 20, 30, and 48 h postinjection. For each microPET scan, three-dimensional regions of interest (ROI) were drawn over the tumor, liver, heart, and muscle on decay-corrected whole-body coronal images. The average radioactivity concentration within a tumor or an organ was obtained from mean pixel values within the ROI volume, which were converted to counts per milliliter per minute by using a conversion factor. Assuming a tissue density of 1 g/mL, the counts per milliliter per minute were converted to counts per gram per minute and then divided by the injected dose (ID) to obtain an imaging ROI-derived % ID/g.

For FDG imaging, the animals were fasted overnight before scanning. After a tail vein injection of 3.7 MBq of $^{18}$F-FDG in 150 μL of PBS, a 7-min prone acquisition scan was done ~60 min after injection. Mice were maintained under isoflurane anesthesia during the injection, accumulation, and scanning periods. PET images were analyzed and quantified as described above.

**Immunotherapy and radioimmunotherapy.** To assess the therapeutic efficacy of cetuximab to HNSCC tumors, inoculated mice with a uniform tumor size approaching ~100 mm$^3$ at 2 to 4 wk after tumor cell inoculation were chosen for study. For SCC1 tumor, four doses of cetuximab (10 mg/kg) were given by i.v. injection every 4 d. The same dose of cetuximab was given to UM-SCC-22B-bearing tumor at an interval of every 2 d, because the tumor growth of UM-SCC-22B is much faster than that of SCC1. To characterize the dose response of $^{90}$Y-cetuximab, animals were randomly divided into three groups and injected with a single dose of 3.7 MBq $^{90}$Y-cetuximab, 3.7 MBq $^{90}$Y-IgG, or saline (n = 7 per group). Tumor growth was monitored by caliper measurement, and tumor volume (assuming an ellipsoidal shape) was approximated by the equation: tumor volume (mm$^3$) = length × width × height / 2.

**Immunofluorescence staining.** Frozen HNSCC tumor slices (5-μm thickness) were fixed with cold acetone for 10 min and dried in air for 30 min. The slices were rinsed with PBS for 2 min and blocked with 10% donkey serum for 30 min at room temperature. The slices were then incubated with cetuximab and rat anti-mouse CD31 antibody for 1 h at room temperature and visualized using FITC-conjugated donkey anti-human secondary antibody (1:200; Jackson ImmunoResearch Laboratories, Inc.) and Cy3-conjugated rat anti-mouse IgG (1:200; Jackson ImmunoResearch Laboratories, Inc.). The slides were observed under a microscope (Axiovert 200M, Carl Zeiss USA), and images were acquired under the same conditions and displayed at the same scale for comparison.

For in vivo staining, tumor samples were collected at 24 h after 50 μg FITC-cetuximab was injected through the tail vein. The tumor sections were fixed and stained with Ki-67 antibody for 1 h at room temperature and visualized using FITC-conjugated donkey anti-human secondary antibody (1:200; Jackson ImmunoResearch Laboratories, Inc.) and Cy3-conjugated rat anti-mouse IgG (1:200; Jackson ImmunoResearch Laboratories, Inc.). The slides were observed under a microscope (Axiovert 200M, Carl Zeiss USA), and images were acquired under the same conditions and displayed at the same scale for comparison.

**Microvascular density measurement.** After CD31 staining, 10 random views in both the center and the periphery of the tumor slices were selected for microvascular density (MVD) analysis using an observer-set threshold to distinguish vascular elements from surrounding tissue parenchyma. Any vessel that contained branching points was counted as a single vessel. The number of vessels counted was divided by the field of view to yield the MVD (as vessels/mm$^2$).

**Ki-67 index measurement.** HNSCC tumor sections were stained with Ki-67–specific SP6 rabbit monoclonal
antibody (Abcam, Inc.) and FITC-conjugated goat anti-rabbit secondary antibody (1:200; Jackson Immunoresearch Laboratories, Inc.) as described above. Nuclear staining of Ki-67 was considered positive. The Ki-67 staining index (SI) was defined as the percentage of positive nuclei within the total number of nuclei in 10 random views, as indicated by 4',6-diamidino-2-phenylindole (DAPI) staining. The positive nuclei were counted by one investigator (X. S.) without prior knowledge of the treatment information.

**Terminal dUTP nick-end labeling assay.** Apoptosis induced by cetuximab treatment was detected by terminal dUTP nick-end labeling (TUNEL) assay using an in situ cell death detection kit (Roche Applied Science) according to the manufacturer's instructions. After fixation and permeabilization, 50 µL of TUNEL reaction mixture were added on the samples. The slides were then incubated in a humidified atmosphere for 60 min at 37°C in the dark. After rinsing with PBS and mounting with DAPI containing mounting medium, the samples were observed under a fluorescence microscope using DsRed channel (excitation/emission maxima, 558 nm/583 nm).

**Statistical analysis.** Quantitative data were expressed as mean ± SD. Means were compared using one-way ANOVA and Student's t test. P values of <0.05 were considered statistically significant.

**Results**

**High expression of EGFR in both SCC1 and UM-SCC-22B cells.** As determined by flow cytometry, both SCC1 and
UM-SCC-22B cells expressed relatively high levels of EGFR using cetuximab as primary antibody (Fig. 1A). EGFR expression on SCC1 cells is significantly higher than on UM-SCC-22B cells. Immunofluorescence staining of tumor sections derived from these two cell lines also showed that there is higher EGFR expression on SCC1 tumor cells than on UM-SCC-22B tumor cells ($P < 0.01$; Fig. 1B).

However, the antibody distribution pattern was very different in these two tumor types. For UM-SCC-22B tumors, the antibody showed a more diffusive and homogenous distribution within the whole tumor region, as indicated by the green fluorescence signals. By contrast, in SCC1 tumors, the antibody only diffused around the perivascular regions and showed more patchy distribution (Fig. 1C).

### Table 1. Biodistribution of $^{64}$Cu activity in HNSCC tumor models

<table>
<thead>
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<th>22B (% ID/g)</th>
<th>SCC1 (% ID/g)</th>
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<tr>
<td>Blood</td>
<td>14.64 ± 1.00</td>
<td>21.60 ± 3.24</td>
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<tr>
<td>Liver</td>
<td>14.86 ± 2.82</td>
<td>17.71 ± 0.56</td>
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<tr>
<td>Muscle</td>
<td>3.43 ± 0.99</td>
<td>3.16 ± 0.45</td>
</tr>
<tr>
<td>Tumors</td>
<td>2.12 ± 0.13</td>
<td>2.12 ± 0.13</td>
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<tr>
<th></th>
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<th>30 h</th>
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<tr>
<td>Blood</td>
<td>6.71 ± 2.17</td>
<td>5.75 ± 2.40</td>
<td>5.50 ± 2.10</td>
<td>11.47 ± 1.22</td>
<td>9.39 ± 0.78</td>
<td>8.67 ± 0.15</td>
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<tr>
<td>Liver</td>
<td>11.17 ± 1.78</td>
<td>10.70 ± 2.02</td>
<td>10.21 ± 2.34</td>
<td>13.28 ± 2.72</td>
<td>12.78 ± 2.20</td>
<td>12.02 ± 3.01</td>
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<tr>
<td>Muscle</td>
<td>3.19 ± 0.83</td>
<td>2.02 ± 0.28</td>
<td>2.34 ± 0.55</td>
<td>2.44 ± 0.22</td>
<td>2.25 ± 0.41</td>
<td>1.22 ± 0.33</td>
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<tr>
<td>Tumors</td>
<td>18.92 ± 1.14</td>
<td>19.07 ± 0.26</td>
<td>21.90 ± 0.98</td>
<td>6.20 ± 0.55</td>
<td>7.35 ± 0.75</td>
<td>10.98 ± 2.97</td>
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NOTE: Data are given as % ID/g and represent mean ± SD of three mice.
Small animal PET imaging of 64Cu-cetuximab tumor delivery. To further characterize the whole body biodistribution and tumor targeting efficiency of the therapeutic antibody in vivo, we did small animal imaging with 64Cu-DOTA-cetuximab on HNSCC tumor-bearing mice. The decay-corrected whole-body coronal images containing the tumors are shown in Fig. 1D for UM-SCC-22B tumors and Fig. 1E for SCC1 tumors. The accumulation of 64Cu-DOTA-cetuximab in UM-SCC-22B tumors was much higher than that in SCC1 tumors at all time points examined after tracer injection. Quantitative data based on ROI analysis are shown in Table 1. At 30 hours postinjection, the UM-SCC-22B tumor uptake of 64Cu-DOTA-cetuximab was 19.07 ± 0.26% ID/g, whereas SCC1 tumor uptake was 7.35 ± 0.75% ID/g. The liver also had prominent radioactivity accumulation, with an uptake of 10.70 ± 2.02% ID/g in UM-SCC-22B tumor-bearing mice and 12.78 ± 2.20% ID/g in SCC1 tumor-bearing mice at 30 hours postinjection, respectively. Blood activity concentration was 5.75 ± 2.40% ID/g and 9.39 ± 0.78% ID/g at 30 hours postinjection, indicating the long circulation lifetime of the antibody.

Therapeutic effect of cetuximab on HNSCC tumors. As shown in Fig. 2A, treatment with cetuximab produced only modest inhibition of cell proliferation on SCC1 cells in vitro as determined by MTT assay. With 50 μmol/L cetuximab, SCC1 cells still kept a survival fraction around 86% compared with control cells. Almost no proliferation inhibition effect was observed in UM-SCC-22B cells even at 120 hours after addition of 50 μmol/L of cetuximab (Fig. 2B). In contrast, cetuximab showed marked inhibition in tumor growth after i.v. injection of four doses of 10 mg/kg body weight in SCC1 tumor-bearing mice (Fig. 2C). As shown in Fig. 2D, however, an agonistic effect on the tumor growth was found in cetuximab-treated UM-SCC-22B–bearing animals. The treatment of cetuximab was well tolerated in both SCC1 and UM-SCC-22B–bearing animals, and no apparent body weight loss was observed (Fig. 2E and F).

Monitoring therapeutic effect by FDG PET imaging. By measuring glucose metabolism, 18F-FDG PET can be used to evaluate the efficacy of therapeutic intervention (30). We carried out 18F-FDG small animal PET scans on SCC1 and UM-SCC-22B tumor models after the third dose of cetuximab treatment. For SCC1 tumors, cetuximab treatment resulted in a significant decrease of FDG uptake within the tumor region, indicating slower tumor growth after treatment (P < 0.01; Fig. 3A and C). By contrast, FDG uptake in UM-SCC-22B tumors showed a significant increase after cetuximab treatment (P < 0.05; Fig. 3B and D), which is consistent with the accelerated tumor growth.

Cetuximab-induced apoptosis and proliferation. To further investigate the mechanism of sensitivity and resistance of HNSCC tumors to cetuximab, we did TUNEL assays to...
evaluate cell apoptosis induced by cetuximab in both SCC1 and UM-SCC-22B tumors. In SCC1 tumors, apoptosis was observed as early as 24 hours after cetuximab treatment and apoptotic cells were found distributed along vasculature. At 48 hours, more apoptotic cells were visualized within the whole tumor region (Fig. 4A). However, for UM-SCC-22B tumors, no apoptosis was observed in nonnecrotic tumor tissue regions, even at 72 hours after cetuximab treatment (Fig. 4B). Based on CD31 staining, we also quantified MVD in cetuximab-treated and control tumors. As shown in Fig. 4C and D, MVD decreased significantly even at 24 hours after treatment ($P < 0.01$). However, there is no significant change of MVD in UM-SCC-22B tumors at 48 hours after treatment with cetuximab, and an increased MVD was observed at 72 hours ($P < 0.05$).

UM-SCC-22B is a fast growing cell line, and inoculated tumors also showed high growth rate. It is not surprising to observe more Ki-67--positive cells in UM-SCC-22B tumors than in SCC1 tumors (SI = 24.01 ± 5.01% versus 11.44 ± 3.93%, $P < 0.01$; Fig. 5). One dose of cetuximab treatment decreased the Ki-67 SI from 11.44 ± 3.93% to 4.73 ± 1.78% and 3.19 ± 1.72% at 24 and 48 hours after therapy, respectively ($P < 0.01$). To the contrary, after a single-dose cetuximab treatment, the Ki-67 SI in UM-SCC-22B tumors increased from 24.01 ± 5.01% to 33.02 ± 2.18% at 48 hours and to 33.34 ± 8.15% at 72 hours ($P < 0.05$).

Radioimmunotherapy with $^{90}$Y-cetuximab. The PET imaging data showed that UM-SCC-22B tumors had very high uptake of cetuximab. To our surprise, cetuximab did not show growth inhibition of UM-SCC-22B tumors but instead showed a proliferation-stimulating effect. We surmised that radioimmunotherapy, by contrast, might be effective in tumor control, due to the high radioactivity dose deposited in the tumor region. In fact, as shown in Fig. 6, in UM-SCC-22B tumor-bearing mice that received an i.v. injection of 3.7 MBq of $^{90}$Y-cetuximab achieved significant tumor growth inhibition. As described above, there is significant passive accumulation of antibody in UM-SCC-22B tumor due to high tumor vasculature and vasculature permeability. Consequently, tumor growth inhibition was also observed after i.v. injection of 3.7 MBq $^{90}$Y-IgG. For SCC1 tumors, $^{90}$Y-cetuximab only produced moderate tumor growth inhibition. At the same time, 3.7 MBq $^{90}$Y-IgG showed no therapeutic effect on SCC1 tumor growth.

**Discussion**

In this study, we have imaged $^{64}$Cu-cetuximab distribution and tumor delivery in HNSCC tumor-bearing mice
with small animal PET. Consistent with our previous observation, UM-SCC-22B tumors showed higher tumor accumulation of imaging tracer, although the EGFR expression was lower than that in SCC1 tumors. SCC1 tumors responded very well to cetuximab therapy, which is consistent with studies done by Huang and Harari (31). However, treatment with cetuximab did not produce any therapeutic effect in UM-SCC-22B tumor growth, but rather accelerated its growth. As reflected by Ki67 staining, UM-SCC-22B tumor proliferation rate increased significantly compared with tumors in the control group at 48 hours after cetuximab treatment. Several possible mechanisms have been suggested to explain the tumor resistance to cetuximab treatment. For example, it has been reported that mutant EGFR, especially EGFRvIII, contributes to the resistance to EGFR-targeted therapy (32). There is no EGFRvIII mutation in SCC1 or UM-SCC-22B cells (Supplementary Fig. S1), however. KRAS mutation and PTEN loss have also been reported to be related to the resistance of colorectal cancers to cetuximab treatment (33, 34). However, PCR results revealed abundant PTEN expression in both tumors (data not shown), and KRAS mutations are rare in HNSCC (35). At 72 hours after cetuximab treatment, MVD increase was obvious in UM-SCC-22B tumors. We speculated that cetuximab might stimulate vascular endothelial growth factor (VEGF) expression, as it is generally believed that anti-EGFR therapy could downregulate VEGF levels (36, 37). However, results from ELISA indicated no significant change in tumoral hVEGF levels or circulating mVEGF levels upon cetuximab treatment in UM-SCC-22B–bearing mice. In addition, hVEGF levels in cell culture media were found to be downregulated by cetuximab in UM-SCC-22B cells, but not in SCC1 cells (Supplementary Fig. S2). VEGF level is thus not directly related to UM-SCC-22B tumor accelerating growth induced by cetuximab treatment. The UM-SCC-22B tumor-stimulating effect of cetuximab will need further investigation.

The $^{64}$Cu-cetuximab accumulation in SCC1 tumors is lower than that in UM-SCC-22B tumors, although SCC1 cells showed higher EGFR expression. As observed in our previous study (28), besides cell surface antigen level, other factors including tumor-specific binding, perfusion, vascularity, vascular permeability, and plasma half-life also influence the final PET imaging quantification when radiolabeled antibody is used as an imaging tracer. In vivo immunostaining with FITC-cetuximab also confirmed the limited perivasculature distribution of cetuximab in SCC1 tumor. It has been reported that the patchy and incomplete tumor perfusion could result in suboptimal therapeutic effects when therapeutic efficacy is dependent upon uniform delivery to tumor cells (38, 39). However, the patchy and limited distribution of cetuximab in SCC1 tumors...
tumors did not hinder therapeutic efficacy. TUNEL assay revealed that at 24 hours after antibody administration, apoptotic cells are localized around vasculature, which is consistent with the antibody distribution. At 48 hours, a more homogenous distribution of apoptotic cells was observed, which might have resulted from antibodies diffusing to greater distances. Another possibility is that low concentrations of antibodies might need a longer time to take effect. Increased tumor cell apoptosis, decreased proliferation, and MVD have been reported in cetuximab-responding tumors upon receiving treatment (40, 41). In this study, we also observed similar therapeutic changes in SCC1 xenografts as early as 24 hours after cetuximab treatment. In addition, FDG PET imaging confirmed decreased metabolism in the SCC1 xenografts following cetuximab treatment.

Although high local antibody delivery did not achieve an inhibitory effect on UM-SCC-22B tumors, we speculated that we may be able to take advantage of the high tumor uptake of antibodies in UM-SCC-22B tumors. As previously reported, the high tumor uptake comes from both specific antibody EGFR binding and passive targeting due to highly vascularized tumor tissue and high vascular permeability (28). In another study (42), we treated a human glioblastoma U87MG tumor model with $^{90}$Y-etaracizumab, a monoclonal antibody against human integrin $\alpha_v\beta_3$. The maximum tolerated dose and dose response analysis in that study revealed that 7.4 MBq per mouse was the optimal therapeutic dose. After comparing the biodistribution data obtained from small animal PET with $^{64}$Cu-etaracizumab (42) and or $^{64}$Cu-cetuximab (43), we chose a one-time dose of 3.7 MBq of $^{90}$Y-labeled cetuximab for radioimmunotherapy in the current study. With this dose, UM-SCC-22B tumor growth was significantly inhibited. Moreover, four of seven treated tumors disappeared, that is, showed a complete response. Even $^{90}$Y-IgG provided a therapeutic effect, because the passive accumulation of IgG in the tumor region deposited sufficient radioactivity. Meanwhile, in SCC1 tumors, we only observed a moderate effect of radioimmunotherapy with $^{90}$Y-cetuximab and no therapeutic effect with $^{90}$Y-IgG. The antibody amount used in radioimmunotherapy was only around 5 $\mu$g/mouse instead of the around 200 $\mu$g/mouse used in immunotherapy with cetuximab. We believe that most of the therapeutic power of $^{90}$Y-cetuximab came from the radiation dose delivered along with the antibodies, but not the antibodies themselves. The 3.7-MBq dose used in this study showed no observable toxicity, as indicated by unchanged mouse body weight (Supplementary Fig. S3). Besides radioisotopes, it should also be promising to conjugate other therapeutic moieties, such as chemotherapeutics, toxins, or molecular therapeutics onto the antibody for targeted delivery, given the dominant tumor uptake of the antibody after systemic administration, as shown in the case of UM-SCC-22B tumors.

According to the studies presented in this report, the efficacy of EGFR-targeted therapy with cetuximab, as determined by small animal PET imaging, is not related to antibody delivery. Another interesting finding is that cetuximab therapy has an agonistic effect on the growth of UM-SCC-22B tumors. Although the underlying mechanism is still unclear, this observation emphasizes the importance of patient selection before providing this therapeutic regimen. Finally, our study revealed limitations of PET imaging with antibodies as tracers to determine antigen expression and to predict tumor response to antibody therapy. However, immuno-PET can provide guidance for targeted therapy using antibodies as delivery vehicles.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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