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Radiolabeling, Characteristics and NanoSPECT/CT Imaging of ^{188}Re-cetuximab in NCI-H292 Human Lung Cancer Xenografts

WAN-I KUO, KAI-HUNG CHENG, YA-JEN CHANG, TSUNG-TSE WU, WEI-CHUAN HSU, LIANG-CHENG CHEN and CHIH-HSIEN CHANG

Isotope Application Division, Institute of Nuclear Energy Research, Atomic Energy Council, Taoyuan, Taiwan, R.O.C.

Abstract. *Background/Aim: Cetuximab has exhibited high EGFR-targeting specificity and clinical promise in previous studies. In this study, we formulated unit dose kits for preparation of high specific activity ^{188}Re-cetuximab for imaging and treatment of EGFR-positive cancer. Materials and Methods: ^{188}Re-cetuximab was prepared by adding 0.37-0.74 GBq/0.5 ml of ^{188}Re-perrhenate for 4 h at 37˚C. Cell surface expression of EGFR, cell binding and cytotoxic effects were evaluated in vitro using both EGFR-positive (NCI-H292, A431) and EGFR-negative (BT483) tumors. A nanoSPECT/CT imaging study was performed in mice bearing EGFR-expressing NCI-H292 tumors. Results: ^{188}Re-cetuximab bound specifically to EGFR-expressing cells and labeling of radionuclides to cetuximab preserved the binding ability of the antibody. Besides, the cytotoxic effect of ^{188}Re-cetuximab was increased dose-dependently. NanoSPECT/CT imaging revealed that ^{188}Re-cetuximab could continually target the tumor region for at least 48 h. Conclusion: The highly specific targeted property of ^{188}Re-cetuximab suggested that it is suitable as a diagnostic tool and maybe a potent radioimmunotherapy agent in EGFR-positive cancers.*

During the past decade, the efficacy of molecular targeted drugs has been proven worldwide, and molecular targeted therapies, including antibodies, have become the mainstream in cancer therapy. Although the major advantages of these targeted molecules are their high tumor specificity and low toxicity profile, not every patient benefits from a specific antibody therapy and may encounter unexpected adverse effects or poor therapeutic effects due to acquired resistance (1, 2). Therefore, the evaluation of the expression of the target molecule in cancer tissues is required to stratify patients, predict therapeutic efficacy, and monitor therapeutic response. Immunohistochemistry, polymerase chain reaction (PCR) and fluorescence *in situ* hybridization (FISH) analyses of biopsy samples are conventional and popular procedures to evaluate expression of the target protein or gene in solid tumors. However, biopsy samples contain tissues from limited number of sections, whereas tumor tissue is heterogeneous. Thus, it is possible that the target expression levels observed in biopsy samples are not representative of that in the entire tumor (3, 4). Moreover, patients often present with multiple lesions and target expression levels can change during treatment. Repetitive biopsies would be required for adequate antigen testing, which in most cases is clinically unfeasible.

Target expression levels and drug distribution can be non-invasively estimated using molecular imaging methods, such as positron emission tomography (PET) and single photon emission computed tomography (SPECT) for both solid and hematological tumors (5, 6). Molecular imaging techniques could overcome the issues associated with biopsies. Advantages include non-invasive screening and visualization of specific membranous target expression in multiple lesions simultaneously. These whole-body scans can be performed repetitively and enable detection of changes in target expression in response to treatment. Therefore, PET and SPECT are very useful in treatment strategies that combine therapeutics with diagnostics, also known as "theranostics."

Epidermal growth factor receptor (EGFR, also known as ErbB-1 or HER-1) expressed on normal human cells has been associated with the control of cell survival, proliferation, and metabolism (7, 8). Higher levels of expression of the receptor have also been shown to be correlated with malignancy in a variety of cancers including colorectal, lung, breast, and head and neck (9-12). Abnormal EGFR activity initiates and promotes mechanisms responsible for tumor growth and progression, including cell proliferation and maturation, angiogenesis, invasion, metastasis, and inhibition of apoptosis. In addition, studies showed that overexpression of EGFR by

Correspondence to: Chih-Hsien Chang, No.1000, Wenhua Rd., Longtan Dist., Taoyuan City, Taiwan 32546, R.O.C. Tel: +886 34711400 ext. 7000, Fax: +886 34711416, e-mail: chchang@iner.gov.tw

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malignant cells is associated with poor prognosis and resistance to therapy (10, 13-15).

Cetuximab, a chimeric human-murine immunoglobulin (IgG1), binds specifically and with high affinity to EGFR on both normal and tumor cells. Binding of the antibody to EGFR prevents stimulation of the receptor by endogenous ligands and results in inhibition of cell proliferation, enhanced apoptosis, and reduced angiogenesis, invasiveness and metastasis (16). Preclinical studies have demonstrated that cetuximab is able to reduce chemotherapy and radiotherapy resistance in EGFR-expressing tumor cells *in vitro* and in nude mice bearing xenografts of human cancer cell lines (17). Moreover, several clinical studies demonstrated cetuximab efficacy in different tumor types and it has been shown to induce response to treatment when used in combination with chemotherapy in patients previously refractory to chemotherapy. Based on these studies, cetuximab is a very promising target to be used in combination with existing therapies or can be conjugated to radionuclides or toxins for the treatment of numerous solid tumors.

In order to monitor tumor localization and its EGFR expression levels in a dynamic fashion using a non-invasive imaging modality, cetuximab was labeled with different radionuclides (18). Since EGFR is overexpressed in a variety of tumors, the accumulation of radiolabeled cetuximab in the tumor cells could serve as companion diagnostic or therapeutic tool. ^{188}Re available from the ^{188}W/^{188}Re generator is a radionuclide used for imaging and therapeutic dual applications. It has a physical half-life of 16.9 h with 155 keV gamma emission (for molecular imaging) and 2.12 MeV β^- emission (for therapeutic radiotherapy) (19). ^{188}Re can be incorporated in the antibody either directly after the reduction of the disulfide bridges or through bifunctional chelating agents. Direct labeling procedures involve the prereduction of protein disulfide bridges, which can be processed by different reducing agents such as 2-mercaptoethanol (2-ME) (20), sodium tartrate (21), ascorbic acid (AA) (22), tris-(2-carboxyethyl)phosphine (TCEP) (23) in order to generate sulfhydryl groups that can be conveniently used to bind [$^{188}ReO_4^-$]. Several studies demonstrated the availability of freeze-dried kits for ^{188}Re radiolabeling of antibodies *via* direct radiolabeling with preserved immunoreactivity (24, 25). Furthermore, the high energy of the β^- emission of ^{188}Re is particularly well suited for effective penetration in solid tumors. In this study, we formulated unit dose kits for preparation of ^{188}Re-cetuximab *via* a direct radiolabeling method for imaging of EGFR-positive cancer.

Materials and Methods

Anti-EGFR lyophilized formulation. Cetuximab was kindly provided by the Taipei Veterans General Hospital and purified by vivaspin to remove drug excipient. Five mg of cetuximab were reduced with 2-mercaptoethanol in a molar ratio of mAb:2-ME=1:550 and purified by size-exclusion PD MidiTrap G-25 column (GE Healthcare Life Sciences, Uppsala, Sweden). Protein concentration was determined following the standard protocol of the Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). 1.125 mg of methylene diphosphonate (MDP), 0.057 mg of $SnCl_2$, 0.0255 mg of ascorbic acid and 0.2 mg of reduced anti-EGFR solution were mixed in a pre-sterilized glass vial and lyophilized for 24 h. All chemicals were purchased from Sigma-Aldrich (Saint Louis, MO, USA) unless otherwise indicated.

Labeling and stability. Cetuximab was labeled with ^{188}Re *via* a direct radiolabeling method using lyophilized kits described previously. Briefly, the ^{188}W/^{188}Re generator was eluted with 0.9% saline and 0.37-0.74 GBq of ^{188}Re-perrhenate in 0.5 ml saline solution were added into a lyophilized kit. Then, 1 N HCl was added to adjust the pH to 5.5-6. After incubation at 37˚C and 100 rpm for 4 h and immediately prior to administration of ^{188}Re-cetuximab to the animal, sterile 0.9% saline was added to the radiopharmaceutical solution, optimizing the final dose to 29.6 MBq /70-100 μl.

To evaluate serum stability, freshly labeled ^{188}Re-cetuximab was analyzed using trichloroacetic acid (TCA) precipitation. The mixture (10 μl) was diluted in a serum sample (190 μl) and incubated for 0, 1, 4, 8, and 24 h at 37˚C. The approach assumes that the TCA precipitable counts represent the majority of intact proteins and those free from the precipitation represent the degree of nuclide detachment and protein degradation. A sample of perrhenate was used as a reference. The analysis was performed in triplicate.

EGFR expression analysis by flow cytometry. NCI-H292, A431, and BT-483 cells were incubated with cetuximab or IgG at 4˚C for 1 h. After washing three times with phosphate-buffered saline (PBS) to remove unbound antibody, the cells were incubated with fluorescein isothiocyanate-labeled anti-human antibody (#555786; BD Bioscience Pharmingen, San Jose, CA, USA) in 1% BSA/PBS at 4˚C for 1 h. Then, cells were washed 3 times by centrifugation at 1,000 rpm for 5 min and resuspended in ice cold PBS. The cell suspensions were analyzed immediately using FACS Calibur and CellQuest software (BD Immunocytometry System, San Jose, CA, USA).

In vitro binding analysis. Cell binding studies with ^{188}Re-cetuximab were carried out using A431, NCI-H292, and BT-483 cells (1×10^6 cells/tube, triplicate). Nonspecific binding was assessed by competition with excess unlabeled cetuximab (0.658 nmol) and performed at 37˚C for 30 min prior to the addition of ^{188}Re-cetuximab. An aliquot of ^{188}Re-cetuximab (23810 Bq, ~68.3 fmol of cetuximab) was incubated with cells at 37˚C for 60 min. Samples were washed three times in cold PBS after centrifugation at 3,000 g for 1 min. Supernatant, which contains the medium in which the cells were incubated and PBS from the washing steps, and pellet for each cell line were counted in a gamma counter (WIZARD 1480, Perkin-Elmer). The percentage of antibody bound to the cells was calculated by (radioactivity of the cell pellet – radioactivity of nonspecific binding)/(radioactivity of the cell pellet + radioactivity of the supernatant) ×100.

Cytotoxicity analysis. Cytotoxicity assay was performed in 96-well plates. Each well was seeded with 1x104 cells and treated with unlabeled cetuximab (~16 μg) or ^{188}Re or ^{188}Re-cetuximab (3.7 MBq/1.6 μg, 18.5 MBq/8 μg and 37 MBq/16 μg), respectively for 4 h. Then, the medium was aspirated and complete medium was added. At 1 day post-treatment, the cytotoxic effects were evaluated by determining cell viability using the alamarBlue assay (DAL1100;

Thermo Fisher Scientific) (26). In brief, 10% volume of alamarBlue reagent was added directly to each well and incubated for 4 h. The fluorescence intensity was measured with excitation and emission wavelengths of 570 nm and 595 nm, respectively. In cytotoxicity assay, the fold change was calculated by dividing the treated group value by the control group value.

Cell culture and tumor xenograft model. Human epidermal A431 cell lines (ATCC) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, and 1% penicillin/streptomycin (P/S). Human lung cancer NCI-H292 cell line (ATCC) was cultured in RPMI medium supplemented with 10% fetal bovine serum, and 1% penicillin/streptomycin. Human breast cancer BT483 cell line (ATCC) was grown in RPMI medium supplemented with 20% fetal bovine serum, and 1% penicillin/streptomycin. All cells were incubated at 37˚C and 5% CO_2. Male BALB/c nude mice were purchased from BioLASCO Taiwan Co., Ltd, Taiwan. The 6-week-old mice were housed in a 12 h light cycle at 22˚C. The animal studies were approved by the ethical review committee in the Institute of Nuclear Energy Research, which followed the NIH guidelines on the care and welfare of laboratory animals. NCI-H292 cells (2×10^6) were subcutaneously (*s.c.*) inoculated into the right thigh of nude mice. Tumors were established for 10 days before tumor imaging.

Tumor imaging in vivo. In order to evaluate distribution of ^{188}Re-cetuximab, single-photon emission computed tomography (SPECT) imaging was performed with four-headed multiplexing multipinhole nanoSPECT (Bioscan Inc., Washington D.C., USA). Subcutaneous tumor mice were intravenously injected with 29.6 MBq of ^{188}Re-cetuximab (n=3) and SPECT images and CT images were acquired using a nanoSPECT/CT scanner system (NanoSPECT/CT PLUS, Mediso, Alsotorokvesz, Budapest, Hungary). Before scanning, mice were anesthetized with 1.5% isoflurine and nanoSPECT/CT images were collected at 4, 24 and 48 h post-injection. The imaging acquisition was accomplished at 60 sec per frame and the energy windows were 155 KeV±20% and 64 KeV±20%. For image reconstruction, the HiSPECT and Nucline software were used for the SPECT and CT images, respectively. The InVivoScrope software was used for fusion of SPECT and CT images.

Data analysis. All data are expressed as mean±standard deviation. Statistical analysis of *in vitro* cytotoxicity assay data was performed with Sigmaplot 12.5 software using the *t*-test. The difference between the compared groups was considered to be statistically significant when $p<0.05$.

Results

Characteristics of ^{188}Re-cetuximab. Labeling efficiency of the lyophilized kit was measured by instant thin-layer chromatography on silica gel-impregnated glass fiber sheets (ITLC-SG) and the result showed that the purity of ^{188}Re-cetuximab was greater than 90% without purification (Figure 1). The radioactivity complex (^{188}Re-cetuximab) remained at the origin (Figure 1A), whereas free ^{188}Re migrated in the strip.^{188}Re-colloid was less than 5% (Figure 1B). Size exclusion-HPLC analysis (Figure 1C) also showed the high radiochemical purity of ^{188}Re-cetuximab. In addition, the radioimmunoconjugates showed high stabilities in serum at 37˚C for 24 h (79.6±2.43%) and in normal saline at room temperature and 4˚C for 24 h (89.05±2.39% and 94.75±3.46%, respectively) (Table I). As shown in Table II, ^{188}Re-cetuximab met all established specifications and the specific activity was 61.97±6.13 mCi/mg of Abs (n=6). In vitro cell binding analysis showed that the cell-bound fraction of ^{188}Re-cetuximab was higher than 60%. Thus, rhenium-188 labeled cetuximab preserved the binding ability of the antibody.

Table I. *In vitro stability of ^{188}Re-cetuximab in various conditions after 0, 1, 4, and 24 h of incubation*.

Incubation time (h)	Normal saline (%)		Rat serum (%)
	4˚C (n=3)	RT (n=3)	37˚C (n=3)
0	95.26±1.68	95.88±1.10	95.31±0.56
1	96.98±1.09	96.70±2.28	92.21±0.64
4	94.79±1.54	95.01±2.45	89.83±1.22
24	94.75±3.46	89.05±2.39	79.60±2.43

Characterization of EGFR expression in various cancer cell lines. Relative expression levels of EGFR in three cell lines was determined by flow cytometry (Figure 2). A relatively high binding of cetuximab was observed in two EGFR-expressing cell lines, NCI-H292 and A431. BT-483 appeared to have the lowest amount of EGFR expression.

EGFR expression levels obtained from the flow cytometry assay were compared with those obtained from the *in vitro* cell binding assay of ^{188}Re-cetuximab. A431 and BT-483 were used as positive and negative control, respectively. The percentage of ^{188}Re-cetuximab bound to the EGFR-positive A431 and NCI-H292 cell lines was 73.4±5.8% and 70.3±3.7%, respectively. BT-483 cell line (0 %) displayed no binding activity (Figure 3). The percentages of ^{188}Re-cetuximab bound to the EGFR-positive cell lines were consistent with the amount of EGFR expression.

Cytotoxicity of ^{188}Re-cetuximab in NCI-H292 cells. Cell viabilities after treatment with different radioactivities of ^{188}Re-cetuximab are shown in Figure 4. At a dose of 18.5 MBq, the percentage of surviving cells decreased to 56.3% in NCI-H292 cells. ^{188}Re-cetuximab showed a radioactivity dose-dependent increase in cytotoxicity, which was most significant at the 37 MBq/well (36.8%) relative to control or cetuximab or free ^{188}Re treated cells ($p<0.05$). The analysis was performed in triplicate.

NanoSPECT/CT imaging of ^{188}Re-cetuximab. To evaluate the *in vivo* specificity and biodistribution of ^{188}Re-cetuximab, nanoSPECT/CT imaging was performed in a NCI-H292 tumor-bearing mice model. NanoSPECT/CT imaging was

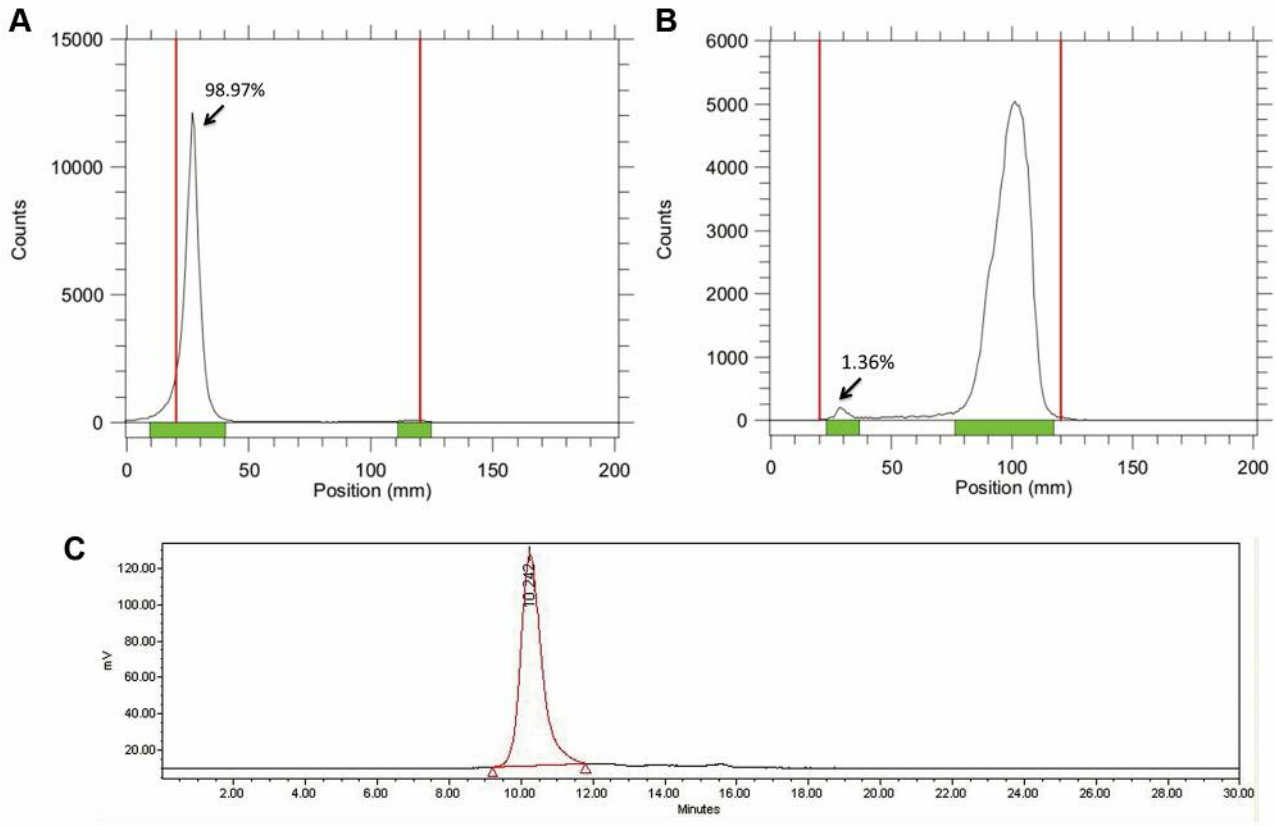

Figure 1. *Radiochemical purity analysis of ^{188}Re-cetuximab by radio-TLC and HPLC systems: Labeling efficiency of the lyophilized kit was measured by instant thin-layer chromatography on silica gel-impregnated glass fiber sheets (ITLC-SG). The result showed that the purity of ^{188}Re-cetuximab was greater than 90% without purification (Figure 1A) and ^{188}Re-colloid was less than 5% (Figure 1B). Size exclusion-HPLC analysis also showed the high radiochemical purity of ^{188}Re-cetuximab (Figure 1C).*

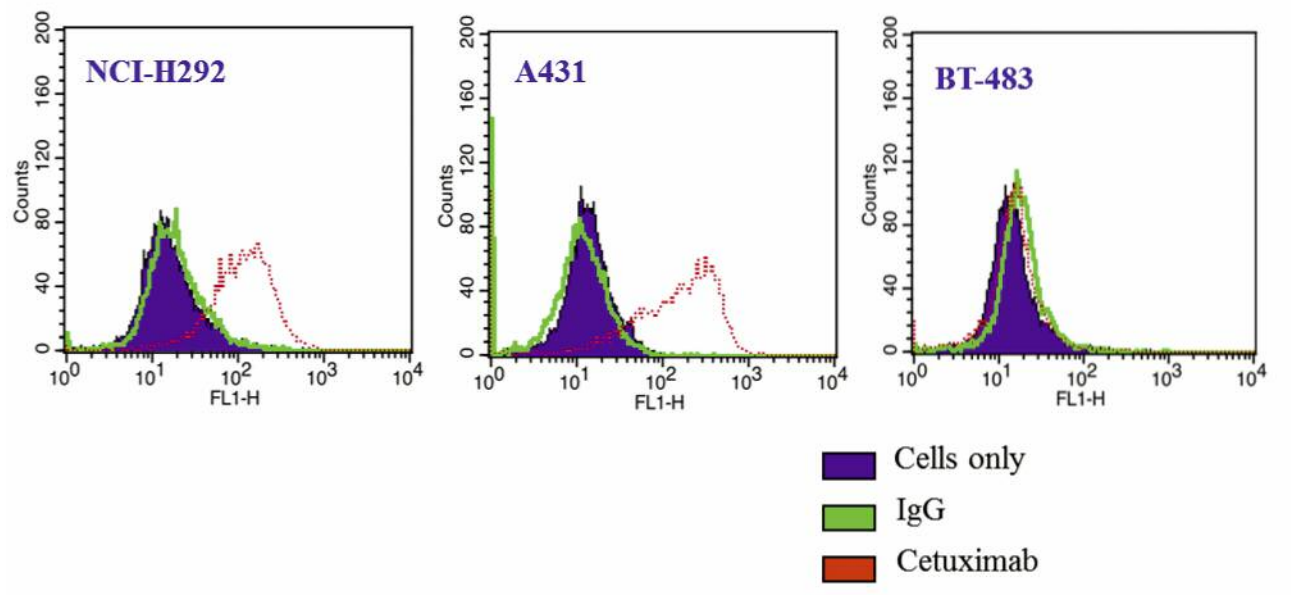

Figure 2. *Flow cytometry analysis of various cell lines for cell surface EGFR expression. Cetuximab displayed a significantly higher binding capacity to EGFR over-expressing cell lines, A431 and NCI-H292, demonstrating the specificity of the antibody.*

Table II. *Quality control tests of the ^{188}Re-cetuximab.*

Quality control test	Acceptance criteria	Results (n=6)
Particulates and color	Clear and colorless	Clear and colorless
pH	Between 6 and 8	6
Radiochemical ID and purity	Rf <0.5; purity ≥90% (Radio-TLC)	R_f=0.076±0.014 Purity=97.32±0.86%
Radiochemical identity	16.9 h	16.9 h
Radiochemical purity	Radiochemical purity ≥90% (HPLC)	96.8±1.61%
Chemical purity	Chemical purity ≥90% (HPLC)	100%
Specific activity	≥1 mCi (37 MBq)/mg of Abs	61.97±6.13 mCi/mg of Abs
Stability (24 h in serum, 37˚C)	purity ≥70%	78.13±2.82%
Cell-bound fraction	≥60%	74.84±5.43%

performed at 4, 24 and 48 h after the intravenous injection of 29.6 MBq of ^{188}Re-cetuximab (12.19 μg of cetuximab). As shown in Figure 5, nanoSPECT/CT images indicated significant accumulation of ^{188}Re-cetuximab in NCI-H292 tumor and ^{188}Re-cetuximab could continually target the tumor region for at least 48 h. In addition, no obvious accumulation was seen in normal tissue at 24 and 48 h post-injection.

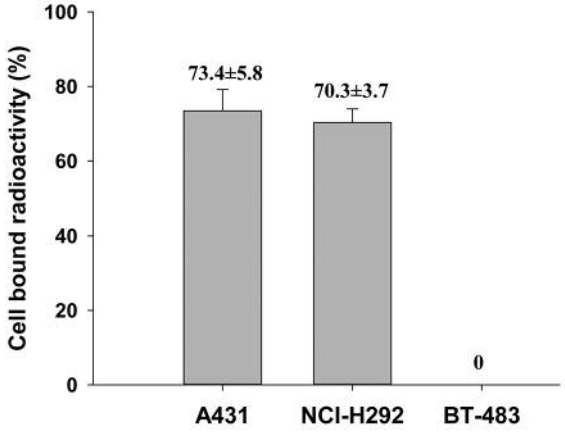

Figure 3. *In vitro ^{188}Re-cetuximab binding to cell lines (A431, NCI-H292, and BT-483). A431 and NCI-H292 cell lines display significant expression of EGFR. The BT-483 cell line displayed no binding activity. The data are expressed as mean in %±SD (n=11 for A431; n=5 for NCI-H292; n=3 for BT-483).*

Discussion

EGFR represents an attractive target for anticancer therapies in a variety of malignant neoplasms, including colorectal, non-small-cell lung, head and neck carcinomas and gliomas. Cetuximab targets EGFR, which plays an important role in proliferation, migration and survival of tumor cells. Knowledge about EGFR levels on and within the cell membrane is important, because only EGFR-depending tumors respond to the therapeutic approaches. Immunohistochemical investigation of tissue slides of the primary tumor are performed to screen for EGFR occurrence in tumor cells. For EGFR, a diagnostic IHC test (EGFR pharmDX Kit) was developed and approved by the US FDA to aid in identifying colorectal cancer (CRC) patients eligible for cetuximab treatment (27). Another development of HercepTest™/Herceptin™ has also been approved as a diagnostic tool for immunotherapy in breast carcinomas. This proceeding represents an important step toward a personalized cancer therapy with major advantages for patients, mainly reduction of toxic side-effects and a dramatically increased efficiency. However, inconsistent methodology and interpretation of EGFR IHC expression possibly lead to false-negative samples (28-30). Also, evaluation of EGFR expression is dependent on storage time of archived tissue sections and a dramatic decline in EGFR staining intensity has been reported with increased storage time of specimens. This may result in EGFR-negative scores in older tissue samples (31). An additional concern is that EGFR expression in metastatic tumor specimens can differ significantly from EGFR expression in the corresponding primary tumor, which is frequently used to obtain the biopsy samples (32, 33). There is an unmet need to develop a non-invasive diagnostic agent, like a radiolabeled imaging agent, to evaluate the receptor expression, both for primary tumors and metastases.

In addition, several mAbs have been approved by the Food and Drug Administration (FDA) for the treatment of cancers. Antibodies specifically target antigens expressed on the cell membrane or ligands and can either be administered unconjugated or conjugated to cytotoxic moieties such as radionuclides or toxins, to increase efficacy. Compared to the

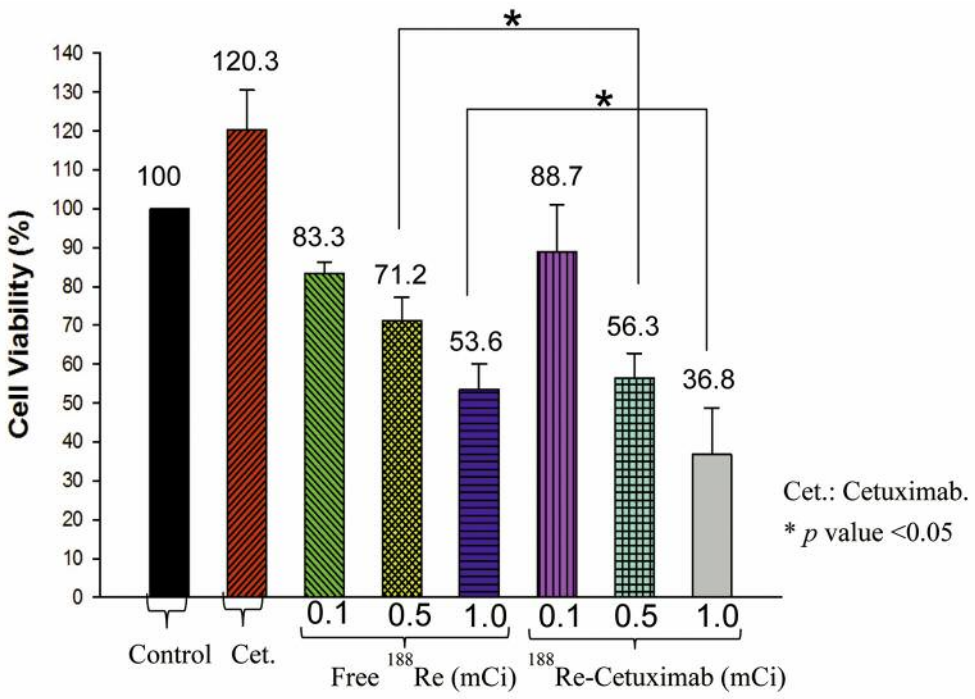

Figure 4. *Cytotoxicity analysis of ^{188}Re-cetuximab. ^{188}Re-cetuximab showed a radioactivity dose-dependent increase in cytotoxicity, which was most significant at the 37 MBq/well (36.8%) relative to control or cetuximab or free ^{188}Re-treated cells (*p<0.05).*

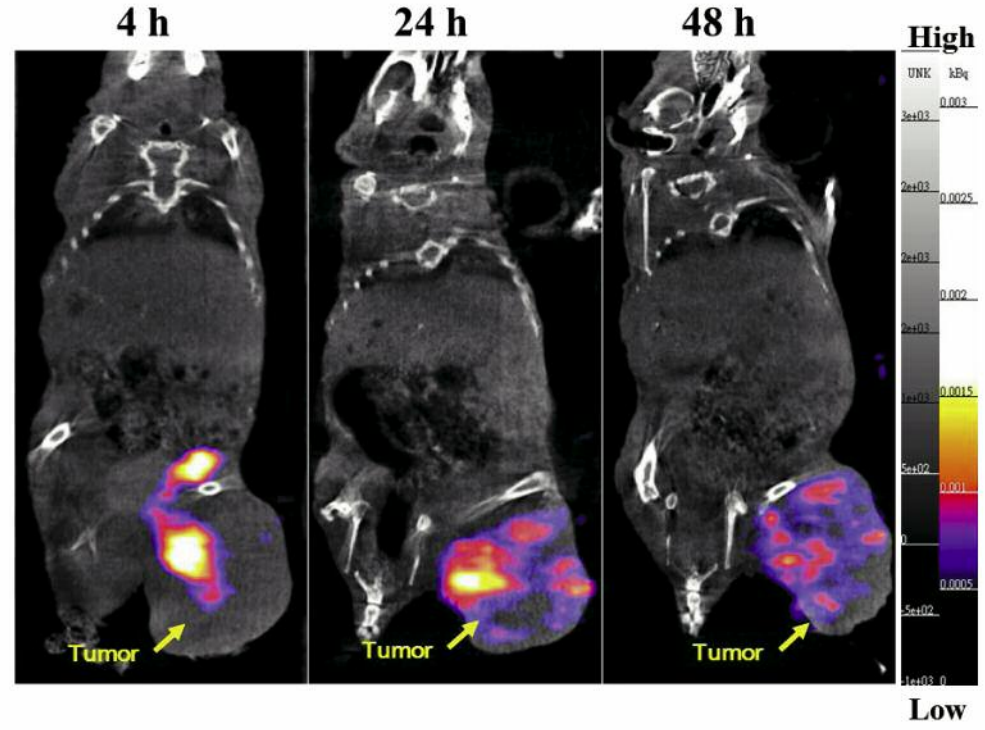

Figure 5. *NanoSPECT/CT imaging of ^{188}Re-cetuximab in the NCI-H292 tumor-bearing mice. Representative whole-body posterior nanoSPECT/CT images of NCI-H292-bearing nude mice at 4, 24, and 48 h after injection of 29.6 MBq of ^{188}Re-cetuximab (12.19 μg of cetuximab). Tumor size is about 1,100-1,500 mm^3. The data indicate that ^{188}Re-cetuximab was a potential theranostic agent for EGFR-positive tumors.*

conventional external-beam radiation therapy, which is used to treat localized disease, radioimmunotherapy offers the possibility to treat localized, metastatic, or diffuse tumors by local or systemic administration of the therapeutic agent (34). Several studies used different diagnostic and therapeutic radionuclides to synthesize cetuximab conjugates. Promising results were obtained with ^{111}In-cetuximab, ^{177}Lu-cetuximab SPECT, ^{64}Cu-cetuximab, ^{89}Zr-cetuximab PET in animal models (35-37).

Generator produced ^{188}Re is carrier-free and it is one of the highest specific activity radionuclides among the currently used β−emitting radionuclides. In addition, ^{188}Re can be produced in the majority of hospitals by an on-site generator in a convenient and inexpensive way. For this, a radiolabeling procedure was designed for the preparation of ^{188}Re-cetuximab. As the ^{188}Re-cetuximab is intended for human use in the future, it possesses characteristics like preservation of the cell binding ability, high radiochemical purity, high specific activity, and highly reproducible quality. In addition, to warrant the safety of involved personnel, safety measurements have to be taken into account. Since the very high labeling efficiency achieved eliminates the need for additional purification, ^{188}Re-cetuximab prepared from the lyophilized kit reduces the manipulation time and minimizes radiation exposure, making it a very competing product.

Conclusion

The anti-EGFR antibody was successfully labeled with ^{188}Re using the direct labeling approach for imaging and radio-immunotherapy. A kit was designed and manufactured for the rapid, simple, and reproducible preparation of ^{188}Re-cetuximab injection. This study showed that the high efficiency, specificity, stability, and convenience of ^{188}Re-cetuximab make it a promising theranostic agent for diagnosis and treatment of EGFR-positive tumors. A further study on the therapeutic efficacy of ^{188}Re-cetuximab was performed to determine its potency.

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