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Original Article

SPECT imaging evaluation of ^{111}indium-chelated cetuximab for diagnosing EGFR-positive tumor in an HCT-15-induced colorectal xenograft

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Abstract

Background: Epidermal growth factor receptor (EGFR) overexpressed in colorectal cancer (CRC) is a tumor target for developing the anti-tumor theranostic agents. Cetuximab, an anti-EGFR monoclonal antibody against EGFR-positive tumors, inhibits cell proliferation and growth was labeled with radioactive ^{111}indium (^{111}In) in this study for diagnosing EGFR-positive CRC. The aim of this study was to evaluate the efficacy of noninvasive nuclear imaging agent ^{111}In-cetuximab and investigate the biological distribution of ^{111}In-cetuximab in the HCT-15-induced EGFR-positive CRC tumor xenografts.

Methods: We conjugated cetuximab with an isotope chelator, diethylene triamine penta acetic acid (DTPA), and consequently labeled cetuximab-DTPA with ^{111}In and measured the labeling efficacy by an instant thin layer chromatography (iTLC). Furthermore, the ^{111}In-cetuximab was investigated and compared for imaging small (50 mm^3) and large (250 mm^3) tumor of CRC xenografts, respectively.

Results: The conjugated ratio between cetuximab and DTPA was 1:6 measured by MALDI-TOF-MS. The better labeling concentration of cetuximab with 10 mCi of ^{111}In was calculated and experimented as 48 μg, resulting in labeling efficacy >80% detected by iTLC. The results revealed that the ^{111}In-cetuximab accumulated in the both sizes of tumors as a reliable noninvasive diagnostic agent, whereas the ratio of tumor to muscle in the large tumor was 7.5-fold. The biodistribution data indicated that the ^{111}In-cetuximab bound to tumor specifically that was higher than that in other organs.

Conclusion: We suggested that the ^{111}In-cetuximab was potential for early diagnosis and prognostic monitor of EGFR-positive CRC in further clinical practice.

Keywords: Cetuximab; Colorectal adenocarcinoma; Epidermal growth factor receptor; ^{111}Indium

1. Introduction

Colorectal cancer (CRC) such as adenocarcinoma occurred with high incidence worldwide belonging to a malignant tumor, which is diagnosed without major symptom at an early stage. Almost 60% of CRC occurs in the developed countries.[1] Since early diagnosis of CRC improves the outcome of

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therapeutic treatment for CRC,[2] it is urgent to develop a reliable and early CRC diagnostic agent. Moreover, CRC is always diagnosed occurring in late stage with metastasis, but the current diagnosing standard, colonoscopy with the histopathologic examination, is insufficient to detect the early tumor and metastasis.

Epidermal growth factor receptor (EGFR) overexpresses in the tissues of CRC by 97% as a cell membrane protein participating in cell proliferation.[3,4] Literature has indicated that EGFR is a tumor target of CRC.[5–7] A previous study has developed EGFR-targeted therapeutic antibodies against CRC such as cetuximab[8] which is a chimeric monoclonal antibody. Besides, cetuximab is also utilized as a targeting probe carrying various diagnostic and therapeutic agents.[9–11] The CRC diagnosis based on the use of cetuximab is proven a promising strategy for noninvasively tracking tumor locations and monitoring the therapeutic effects.

Compared to the gold standard, colonoscopy with histopathologic examination, in diagnosing CRC, the noninvasive nuclear imaging techniques such as single-photon emission computerized tomography/computer tomography (SPECT/CT) or positron emission tomography/computer tomography (PET/CT) provide higher sensitive diagnostic resolution and wide-screen for whole tissues in body specimen,[12–14] suggesting that early tumor diagnosis and metastasis diagnosis by nuclear imaging techniques are feasible. Based on the concept of nuclear imaging methodology, this study aimed to evaluate the noninvasive nuclear imaging technique for diagnosing EGFR-positive CRC using a radioactive isotope-chelated-cetuximab, which may provide an evidence for the consequent utilization of EGFR-specific anti-tumor therapy.

In this study, we intended to label cetuximab with ^{111}indium (^{111}In) (half-life = 2.83 days, *r*-ray = 0.2454 MeV) through diethylene triamine penta acetic acid (DTPA) chelator. The optimal labeling ratio was calculated and experimentally investigated. The cell binding of cetuximab-DTPA to EGFR-positive HCT-15 cells was investigated. The reliability of nuclear imaging diagnosis using ^{111}In -cetuximab was demonstrated in the HCT-15-induced tumor xenografts which carrying small (50 mm^3) and large (250 mm^3) tumors individually. Furthermore, the biodistribution of ^{111}In-Cetuximab in tumor xenografts was also investigated.

2. Methods

2.1. HCT-15 culture and tumor xenograft model

Human colorectal carcinoma cells (HCT-15) were purchased from the American Type Culture Collection (ATCC) and cultured in F12K medium with 10% of fetal bovine serum. HCT15 is an EGFR-positive colorectal cancer cell line with KRAS mutation[15] which model is similar to a previous study utilizing HCT116 for EGFR-nuclear imaging.[10] All cells were incubated at 37 °C and 5% CO_2. Male nude mice were purchased from BioLASCO Taiwan Co., Ltd, Taiwan. The 5-week-old mice were housed in a 12 h-light cycle at 22 °C. The animal studies were approved by the institutive ethical review committee in Institute of Nuclear Energy Research, Taiwan, which followed the NIH guidelines on the care and welfare of laboratory animals. HCT-15 cells (2×10^6) were subcutaneously (s.c.) inoculated into the right leg of nude mice. Tumors were established for 7 days as the small tumor model (50 mm^3), and 30 days as the large tumor model (250 mm^3) before the tumor imaging.

2.2. Conjugation and measurement of cetuximab-DTPA

To create cetuximab-DTPA, cetuximab was incubated with *P*-SCN-Bn-DTPA (w/w 1:10, Macrocyclics, Dallas, TX, USA) in carbonate-bicarbonate buffer (pH 9.0) at room temperature for 2 h. The cetuximab-conjugated DTPA was purified using G-25 column, whereas the second ml was collected. The cetuximab and cetuximab-DTPA were added with an equal volume of sinapinic acid (20 mg/ml in 50% acetonitrile/0.5% TFA) and dried on a steel plate. The molecular weights of antibodies were analyzed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS, UltraflexIII, Bruker Daltonics GmbH, Germany).

2.3. Cetuximab-DTPA binding assay

Each HCT-15 cells (2×10^6 cells) were treated with 1 μg/ml of cetuximab-FITC for 30 min at room temperature. The 20-fold of cetuximab or cetuximab-DTPA higher than cetuximab-FITC was added simultaneously for investigating the competing capacity. After the reaction, the medium was removed and washed using phosphate-buffered saline (PBS) for three times. The cells in PBS buffer were analyzed using FACSCalibur Flow Cytometer (BD Bioscience, USA).

2.4. The labeling of ^{111}In-cetuximab in vitro

First, the cetuximab-DTPA was mixed and incubated with ^{111}In by 1:1 molar ratio. The specific radioactivity (SRA) of ^{111}In was calculated as following formula: SRA (Bq/g) = λN = 0.693/half-life of ^{111}In (seconds) x 6.03×10^{23}/MW = 1.51351×10^{16}. Therefore, 10 mCi of ^{111}In = 3.7×10^8 Bq/1.51351×10^{16} = 2.445×10^{-2} μg. According to the calculated results, the cetuximab-DTPA incubated with 10 mCi of ^{111}In was 2.445×10^{-2} μg/111×156176 = 34.4 μg by 1:1 molar ratio. Since the conjugated ratio between cetuximab and DTPA was measured 1:6, we expected that 5.73 μg of cetuximab could be labeled with 10 mCi of ^{111}In molecules. In experiments, we incubated 3, 6, 12, 24, 48, 96, 192, 384, and 768 μg of cetuximab-DTPA with 10 mCi of ^{111}In, respectively, for 30 min and 24 h in PBS buffer, pH7.4. This experiment was performed just once for reason of as low as reasonably achievable (ALARA), however, the labeling rate was measured every time when applying in animal nuclear imaging. The labeling rate >80% was acceptable as performed in the tumor xenografts. The labeling efficiency was measured using instant thin layer chromatography (iTLC) on the silica gel impregnated glass fiber sheets (PALL corporation, USA), whereas PBS was used as the

mobile phase. Then, the sheets were measured using a radioactive scanner (AR-2000radio-TLC Imaging Scanner, Bioscan, France).

2.5. Nuclear imaging and biodistribution in HCT-15-induced tumor xenografts

The HCT-15-induced tumor xenografts were intravenously injected with ^{111}In -cetuximab ($n = 3$) or ^{111}In alone ($n = 3$) by 1 mCi of radioactivity for each mouse. A Nano-SPECT/CT (Mediso Medical Imaging Systems, USA) was utilized to detect and image the tumors in the tumor model *in vivo*. For investigating the biodistribution of ^{111}In-cetuximab in EGFR-positive HCT-15-induced xenografts, the organs were harvested and measured the radioactivity using a gamma counter (1470 WIZARD, PerkinElmer, USA) after the agents and ^{111}In injection for 48 h. The percent injected dose per gram of tissue (%ID/g) was utilized to represent the radioactive intensity in each collected organ.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism V5.01 software (GraphPad Software, Inc., California, USA). All analysis data with more than two groups were performed by ANOVA followed by posthoc analysis with Bonferroni's test. Student's *t*-test was used to compare two groups. The significance difference was acceptable as $p < 0.05$.

3. Results

3.1. Cetuximab specifically bound to EGFR-overexpressed HCT-15 cells

To investigate EGFR expression in colorectal HCT-15 cancer cells, cetuximab was labeled with fluorescent FITC, and then the agent was purified using a G-25 column. HCT-15 cells were treated and incubated with none, 1 μg/ml of cetuximab-FITC, or 1 μg/ml of cetuximab-FITC plus 10 μg/ml of cetuximab, respectively, for 30 min at room temperature. We figured out that the fluorescent intensity in the cetuximab-FITC group was higher than that in other groups (Fig. 1A and B), revealing that cetuximab specifically bound to HCT-15 cells which overexpressed EGFR.[15]

3.2. Cetuximab-DTPA bound to HCT-15 cells

Cetuximab was labeled with ^{111}In through DTPA chelator, therefore, we first conjugated cetuximab with DTPA. We added excess DTPA to cetuximab by 10-fold for maximally conjugating DTPA to available amide groups of cetuximab. MALDI-TOF MS results showed that the molecular weight 152,153Da of cetuximab shifted as 156,176Da (Fig. 2A), indicating that the conjugated ratio was approximately 1:6 between cetuximab and DTPA. In order to investigate the binding capacity of cetuximab-DTPA to HCT-15 cells, 10 μg/ml of cetuximab-DTPA was added with 1 μg/ml of cetuximab-FITC as a competitor. We found that cetuximab-DTPA reduced the binding capacity of cetuximab-FITC to HCT-15 cells (Fig. 2B and C), revealing that cetuximab-DTPA still had the binding capacity to EGFR on HCT-15 cells.

3.3. The optimal labeling ratio of cetuximab-DTPA with ^{111}In

The theoretical labeling concentration of cetuximab-DTPA with ^{111}In is 5.73 μg of cetuximab-DTPA to 10 mCi of ^{111}In described in Methods and Materials. We intended to evaluate the accurate labeling ratio experimentally, 3, 6, 12, 24, 48, 96, 192, 384, 768 μg of cetuximab-DTPA were incubated with 10 mCi of ^{111}In, respectively. We found that the amount of cetuximab-DTPA over 48 μg resulted in >80% labeling efficiency (Fig. 3A and B), indicating that the optimal labeling concentration was 48 μg of cetuximab-DTPA to 10 mCi of ^{111}In corresponding to the theoretical calculation. The labeling efficiency of ^{111}In-cetuximab was measured >80% in 24 h (Fig. 3A), revealing that ^{111}In -cetuximab was stable.

3.4. ^{111}In-cetuximab nuclear imaging and biodistribution

Since ^{111}In-cetuximab was created, we applied ^{111}In-cetuximab to detect the EGFR-positive tumors in the HCT-15-induced xenografts. The ^{111}In-cetuximab was injected in the tumor models implanted with a small tumor (50 mm^3) and large tumor (250 mm^3) and consequently imaged using a Nano-SPECT/CT device. We found that ^{111}In-cetuximab accumulated in the mouse liver and tumor in 24 h poster injection, including small and large tumors (Fig. 4A and B). Otherwise, ^{111}In accumulated in kidney majorly. The radioactivity of ^{111}In-cetuximab in the tumor was higher in small or large tumor model compared to control group. Next, the biodistribution of ^{111}In-cetuximab was investigated compared to that of ^{111}In. The results were consistent with the SPECT/CT images showing the higher radioactivity in tumors compared to other organs in the ^{111}In-cetuximab-injected large tumor xenografts (Fig. 5B). Otherwise, ^{111}In majorly distributed in the kidney (Fig. 5A). The tumor to muscle ratio of ^{111}In -cetuximab was measured 7.5-foldwhich was higher than that of ^{111}In group measured as 3.1-fold (Fig. 5C), indicating that ^{111}In-cetuximab specifically bound to EGFR-positive tumors as a reliable diagnosing agent. Meanwhile, the sum of radioactivity in ^{111}In group was higher than that in the ^{111}In-cetuximab group (Fig. 5D). The result indicated that ^{111}In labeled with cetuximab through chelator DTPA was easily excreted out the mice better than free ^{111}In, suggesting that this labeling method may not lead to accumulation of ^{111}In metal in mice.

4. Discussion

EGFR overexpresses in a variety of cancers, including CRC,[3,16] head and neck cancer,[17] lung cancer.[18,19] Therefore, diagnosing the EGFR-positive tumor is an important issue for

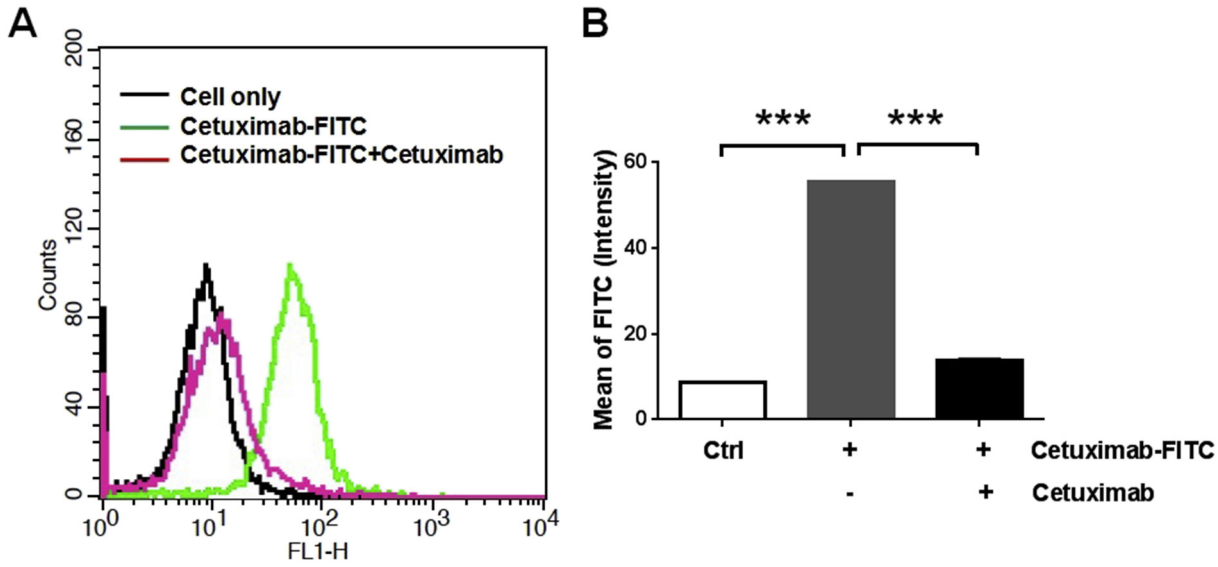

Fig. 1. **Overexpression of EGFR was recognized by cetuximab in colorectal HCT-15 cells.** To make sure the overexpression of EGFR in HCT-15 cells, and the binding capacity of cetuximab to HCT-15 cells, cetuximab-FITC was utilized and incubated with HCT-15 cells. (A & B) The higher binding capacity was found in cetuximab-FITC (1 μg/ml) group measured using a flow cytometry technique, which was blocked when the 10-fold of cetuximab was added. ***$p < 0.001$.

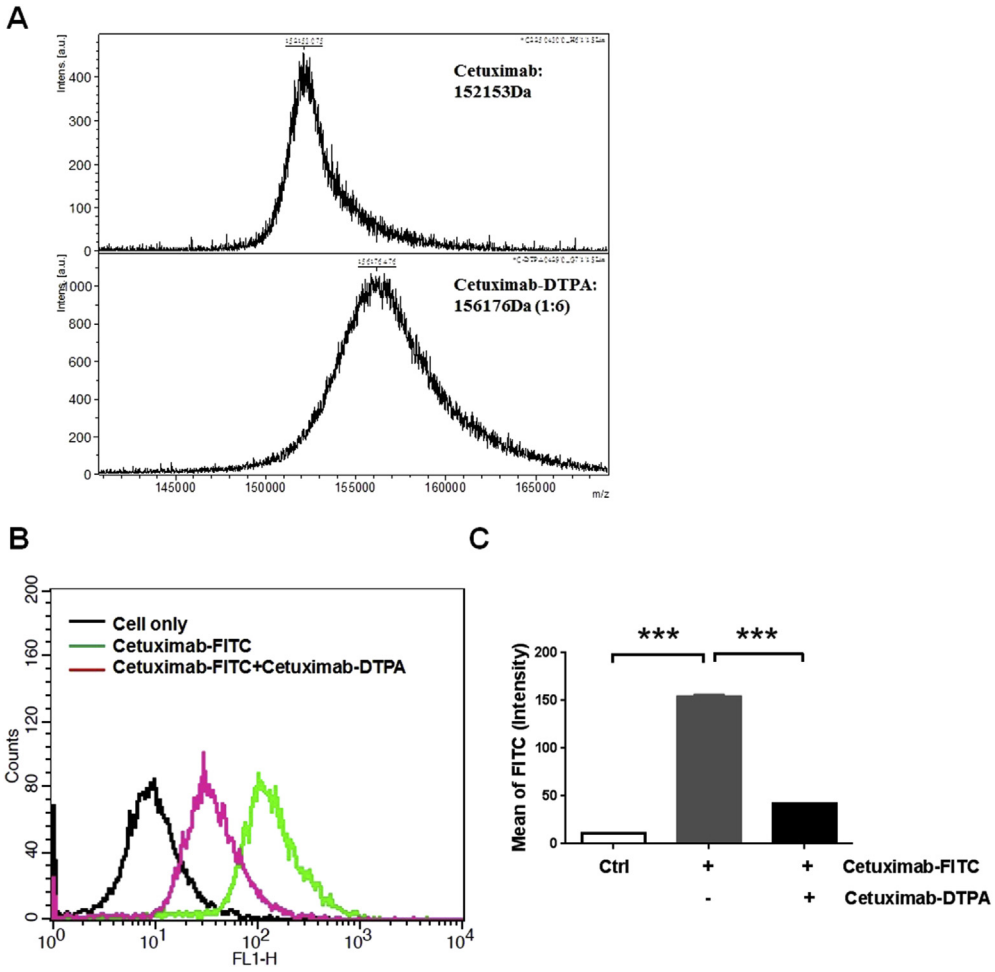

Fig. 2. **The ratio of cetuximab to DTPA was 1:6.** (A) Cetuximab was conjugated with *p*-SCN-Bn-DTPA for exact 2 h, which was measured using MALDI-TOF MS. The molecular weight of cetuximab was measured 152,153Da and shifted as 156,176Da after DTPA (649.9Da) conjugation. The ratio was estimated 1:6 between cetuximab and DTPA. (B&C) The cetuximab-DTPA was used as a competitor against cetuximab-FITC for determining the binding capacity of cetuximab-DTPA. The 10-fold of cetuximab-DTPA significantly blocked the binding capacity of cetuximab-FITC to HCT-15 cells detected using a flow cytometry technique. ***$p < 0.001$.

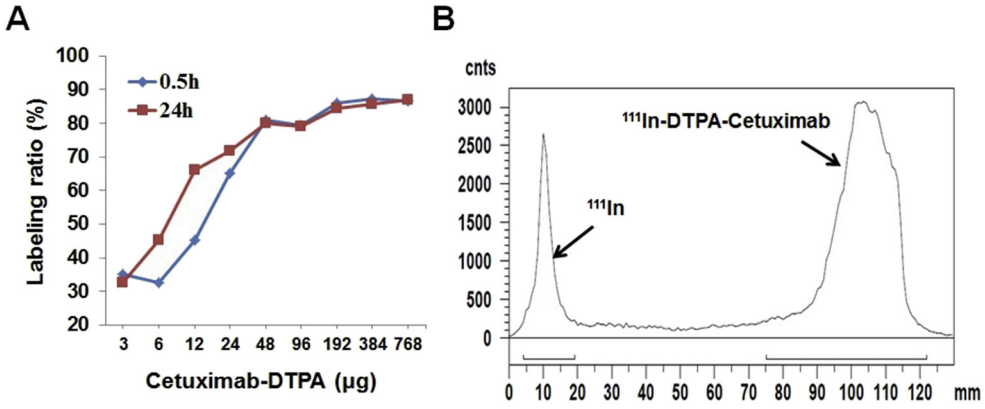

Fig. 3. **Labeling of cetuximab-DTPA with radioactive ^{111}In.** Theoretically, 10 mCi of ^{111}In was labeled with 34.4 μg of cetuximab by 1:1 molar ratio described in Methods and Materials. (A) In the experiment, a wide range of cetuximab-DTPA (3–768 μg) was incubated with 10 mCi of ^{111}In, respectively. The labeling efficiency was determined using iTLC. The higher amount of cetuximab-DTPA increased the labeling efficiency, but the limit was around 80% shown by the arrow whereas 48 μg of cetuximab-DTPA was incubated with 10 mCi of ^{111}In both for 0.5 h or 24 h. (B) The iTLC result indicated that 48 μg of cetuximab-DTPA were incubated with 10 mCi of ^{111}In for 0.5 h demonstrated the successful labeling.

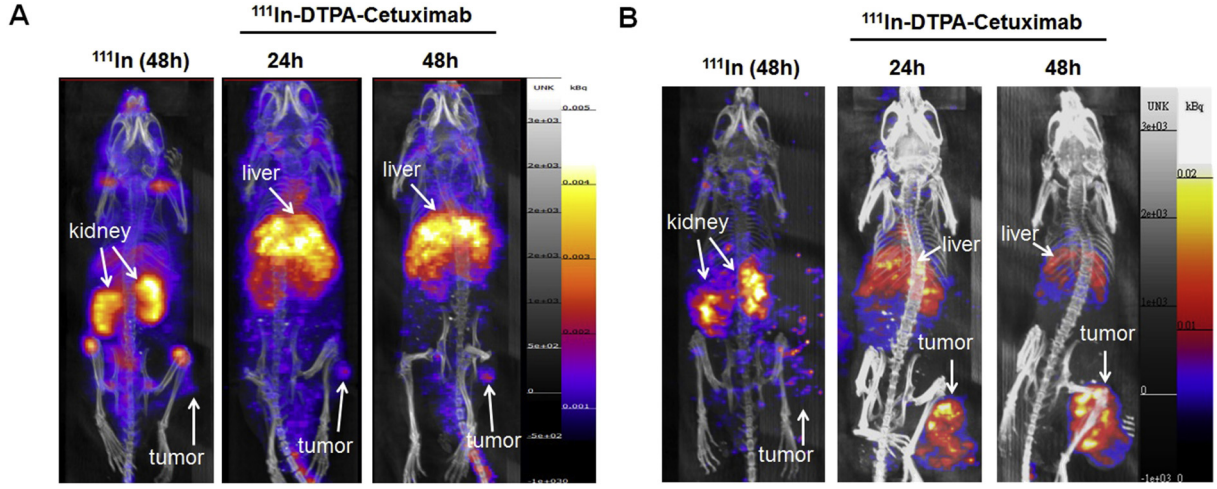

Fig. 4. **^{111}In-cetuximab was utilized to diagnose early and advanced colorectal cancer in HCT-15-induced xenografts.** (A) The early tumors ($n = 3$) implanted in nude mice for 1 week (50 mm^3) were detected using ^{111}In-cetuximab cooperated with SPECT/CT. The radioactive image in the tumor of the ^{111}In-cetuximab group was apparently observed both in 24 h and 48 h and higher than that in the ^{111}In group. ^{111}In-cetuximab majorly accumulated in liver and tumor, otherwise, ^{111}In accumulated only in the kidney. (B) The advanced tumor (250 mm^3, $n = 3$) was established and imaged. The tumors in the ^{111}In-cetuximab group were apparently detected and imaged, and radioactive signals were higher than that in the ^{111}In group.

selecting the adequate therapy. In this study, we intended to label cetuximab with radioactive ^{111}In and to optimize the labeling condition for creating ^{111}In-cetuximab serving as a diagnostic imaging tool for CRC. Our results demonstrated that ^{111}In-cetuximab specifically targeted to EGFR-positive HCT-15-induced tumors, and the optimal labeling concentration was 48 μg of cetuximab with 10 mCi of ^{111}In. This research provided the manufactured condition for EGFR nuclear imaging agent, ^{111}In-cetuximab.

Targeting to EGFR based on its specific antibody is useful and potential for developing the diagnostic methodology for EGFR-positive tumors. Since utilization of radiolabeled cetuximab has been applied for diagnosing tumors such as CRC[20] or head and neck tumor,[21] to investigate and optimize the labeling ratio between cetuximab and isotope was needed. Although the labeling efficiency was limited around 80% when using 48 μg of cetuximab incubated with 10 mCi of ^{111}In, the EGFR-positive tumor was clearly imaged and diagnosed. The higher amount of cetuximab with 10 mCi of ^{111}In led to increased labeling efficiency, however, the unlabeled cetuximab played as a competitor blocking the binding of ^{111}In-cetuximab similar to a study published by Nayak et al. using co-injection of cetuximab with ^{86}Y-labeled cetuximab.[22] Therefore, finding the optimal radio-labeled rate is necessary for improving the radio-imaging signals. Previously, Shih et al. have utilized 100 μg of cetuximab to label with 10 mCi of ^{111}In, resulting in similar radio-labeled rate (~80%).[10] Although the radio-labeled rate >80% is acceptable for a nuclear imaging application, the extra unlabeled cetuximab may reduce the radioactive signals in real practice, leading to lower resolution of nuclear imaging. According to their data, they have declared that ^{111}In-cetuximab leads to highest

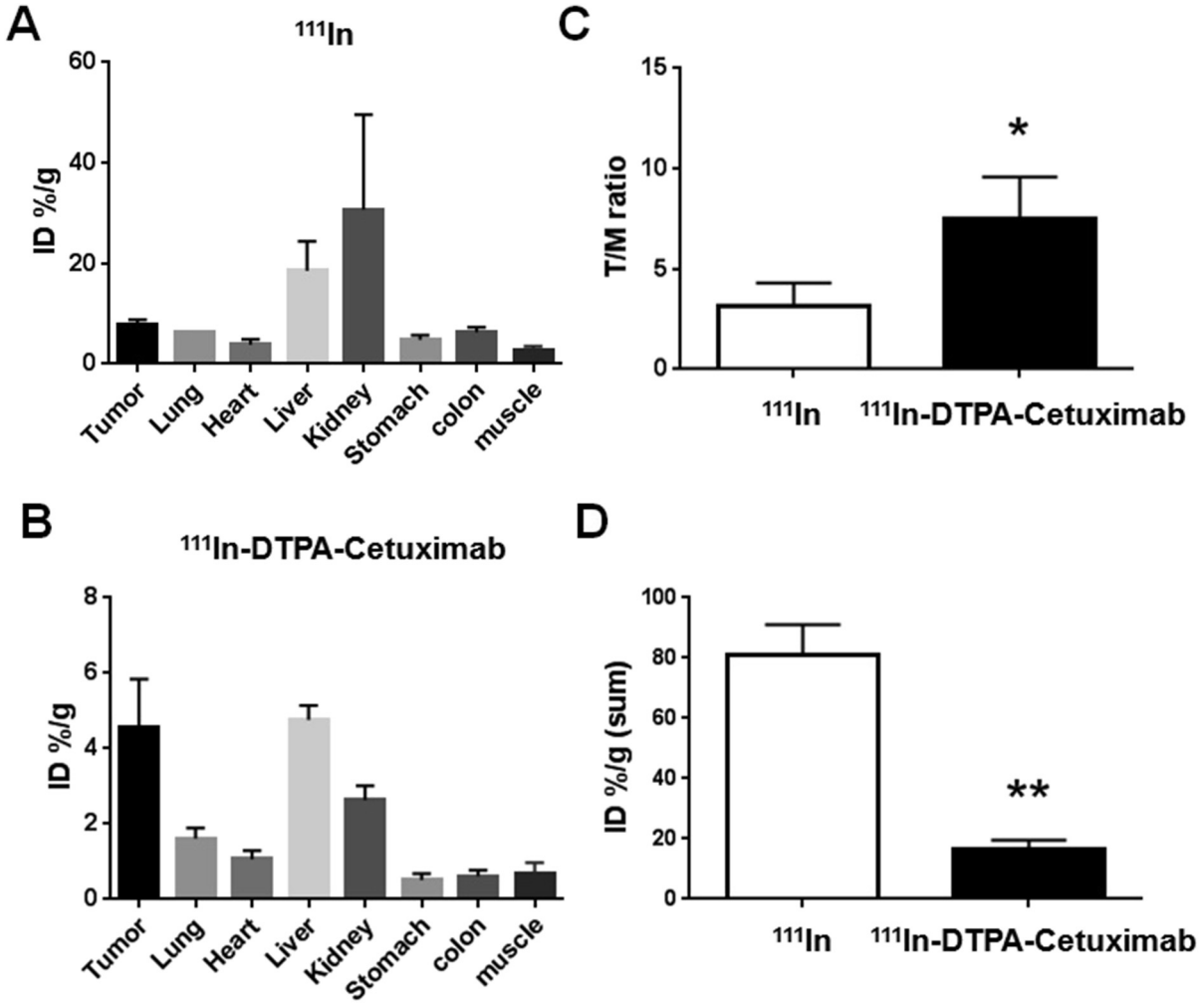

Fig. 5. **Biodistribution of ^{111}In-cetuximab in the HCT-15-induced xenografts.** (A) Biodistribution of ^{111}In in tumor xenografts was investigated. The results indicated ^{111}In accumulated highest in kidney and liver rather than that in the tumor. (B) The result revealed that ^{111}In-cetuximab apparently targeted and accumulated in tumor higher than that in other organs except for liver. (C) The tumor to muscle (T/M) ratio was compared and investigated. The T/M ratio of ^{111}In-cetuximab was higher than that of ^{111}In. (D) The sum of radioactivity in the collected organs was significantly reduced in the ^{111}In-cetuximab group compared to ^{111}In group, indicating that ^{111}In-cetuximab rapidly excreted from the tumor xenograft mice. *$p < 0.05$. **$p < 0.01$.

radioactive signals in 72 h after injection by tail vein. However, we not only detected the HCT-15-derived large tumors in 24 h but also detected the small tumors, implying the optimal labeling ratio was significant for nuclear imaging. Therefore, in order to obtain a better and higher radioactive imaging, we suggested that the labeling ratio: 48 μg of cetuximab with 10 mCi of ^{111}In was adequate.

Currently, cetuximab has been labeled with ^{111}In,[10, 23] ^{89}Zr,[24, 25] ^{64}Cu,[26,27] and ^{99m}Tc.[28] The half-life of ^{99m}Tc is 6 h, which is not enough applied in antibody-based nuclear imaging such as cetuximab with the apparent imaging signals after 24 h injection. ^{89}Zr and ^{64}Cu are PET isotopes having higher resolution than SPECT imaging such as images derived from ^{111}In.[29] ^{64}Cu (half-life: 12.7 h) is potential for applying not only in diagnostic imaging but also for targeted radiotherapy due to the additional β^- particles emitting. Because the half-life of circulation of antibody in the biologic body was over 63 h, and the highest imaging intensity of antibody appeals after 48 h injection, ^{89}Zr (half-life: 3.3day) and ^{111}In (half-life: 2.8day) are more suitable to label with cetuximab. No matter what radioactive isotopes are selected to label with cetuximab, the optimal labeling ratio is equivalent to the result demonstrated in this study.

^{111}In-cetuximab was demonstrated to diagnose an early small tumor and advanced large tumor in this study. Due to the labeling of isotope was through DTPA chelator, further radiotherapy using β^--emitted yttrium-90 (^{90}Y through DTPA) chelating is feasible.[30–32] ^{90}Y-cetuximab combined with external irradiations had been demonstrated to reduce tumor size in a 3D cell assay.[33] Moreover, ^{111}In-cetuximab may be used to diagnose the prognosis of chemotherapy, tumor metastasis, and the cetuximab-derived resistance. This study addressed and evaluated the correct labeling ratio, nuclear imaging, and biodistribution of ^{111}In-cetuximab.

Beside tumors, ^{111}In-cetuximab was also highly accumulated in the liver which also overexpresses EGFR.[34] The biodistribution results indicated the equal radioactive intensity between tumor and liver in a large tumor model in 48 h. Since

existed location of the colon is distinguished from the liver, the nuclear imaging of CRC using ^{111}In-cetuximab may be not interfered by that from the liver. However, the acute toxicity in the liver is needed to be monitored in the performance of ^{111}In-cetuximab, particularly in the patients with liver diseases. Moreover, for detecting the tumor metastasis in the liver, this nuclear imaging technique based on ^{111}In-cetuximab is inadequate. Other techniques are needed for assisting the diagnosis of tumor metastasis in liver.

In conclusion, we evaluated that ^{111}In-cetuximab was useful for detecting an EGFR-positive tumor, and optimized the labeling ratio between cetuximab and radioactive ^{111}In. The theoretically labeling ratio was equivalent to the experimental result, which may be applied in another labeling pair of antibody and isotope. We suggest that optimal labeling of ^{111}In-cetuximab can be used to diagnose EGFR-positive CRC.

Acknowledgments

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