Preparation and biological evaluation of ^{166}Ho-DTPA-Cetuximab

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Preparation and biological evaluation of ^{166}Ho-DTPA-Cetuximab

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ABBREVIATION

DOTA : tetraazacyclododecane tetraacetic acid

DTPA : diethylene triamine pentaacetic acid

EGFR : epidermal growth factor receptor

Ho : holmium

I : iodine

ID/g : injected dose per gram

ITLC-SG : instant thin layer chromatography - silica gel

RIT : radioimmunotherapy

ABSTRACT

Preparation and biological evaluation of ^{166}Ho-DTPA-Cetuximab

Holmium-166 (^{166}Ho) has decay characteristics (γ: 0.081 MeV, 1.38 MeV for radiodetection, β^-: 1.78 MeV, 1.84 MeV for radiotherapy, half-life: 26.8 hr) suitable for radioimmunotherapy (RIT) of cancer. Cetuximab, a chimeric monoclonal antibody against epidermal growth factor receptor (EGFR), is currently used to treat several solid tumors. For evaluating the feasibility of radioimmunotherapy, ^{166}Ho-diethylene triamine pentaacetic acid (DTPA) -Cetuximab was prepared and its biological characteristics were examined in an EGFR-positive tumor model. To evaluate EGFR expression levels in five different tumor cell lines (A431, A549, SNU-C2A, U87MG, and CT-26 cells), *in vitro* cell binding assay was performed using ^{125}I-Cetuximab. The EGFR expression in A549 tumor cells was confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR). Cyclic DTPA anhydride was conjugated to Cetuximab with six different molar ratios of DTPA to Cetuximab (50:1, 100:1, 200:1, 300:1, 500:1 and 1000:1). The DTPA-Cetuximab conjugate prepared at the 1,000:1 ratio exhibited the highest number of bound DTPA

(88.4 molecules). The ^{166}Ho-radiolabeling yield was also the highest in the same DTPA-Cetuximab conjugate (98.9%). The immunoreactivity and specific activity of the ^{166}Ho-DTPA-Cetuximab was 60.7 ± 3.0% and 94.0 MBq/mg, respectively. The ^{166}Ho-DTPA-Cetuximab conjugate was stable in the presence of serum proteins (>95% for 24 hr at 37℃). In A549 xenograft mice, ^{166}Ho-DTPA-Cetuximab showed the highest tumoral uptake (9.1 ± 1.8% ID/g) at 72 hr post injection. The conjugate also showed the highest tumor-to-blood (9.1 ± 2.9) and tumor-to-muscle ratios (9.1 ± 3.7) at 144 hr post injection, respectively. The selective localization of ^{166}Ho-DTPA-Cetuximab in EGFR-positive A549 tumor xenografts was confirmed by gamma camera imaging at 24 hr,. These data showed that the ^{166}Ho-labeled immunoreactive DTPA-Cetuximab conjugates with a high specific activity would be a renovated modality of radioimmunotherapeutics for EGFR-expressing tumors.

Keywords : Cetuximab, ^{166}Ho, DTPA, radioimmunotherapy

I. INTRODUCTION

Radioimmunotherapy has been utilized as nuclear medicine for tumor therapy [1,2,3]. In radioimmunotherapy, monoclonal antibodies with selectivity for the target cells or tissues are linked to radionuclides with high beta (iodine-131 and yttrium-90) or alpha (bismuth-213 and astatine-211) emitters resulting in cell death. In general, beta particles, with a penetration range of millimeters, are suitable for target-specific tumor therapy, whereas alpha particles, with a penetration range of a few cell diameters, are suitable for micrometastasis or circulating tumor cells [1,4].

Cetuximab (MERCK, Germany), an antibody being used therapeutically to target epidermal growth factor receptor (EGFR) [8,9], was originally reported to stimulate EGFR internalization and downregulation [10]. Also, Cetuximab (either alone or in combination with drugs or radioisotopes) has been used for treatment of several solid tumors, such as non-small cell lung carcinoma, pancreatic cancer, head and neck cancer, etc. [11,12,13].

The radiolanthanides (ie., samarium-153, lutetium-177, holmium-166, etc.) are considered as excellent candidates for radiotherapy because their desirable physical characteristics and availability. The various radiolanthanides exhibit a variety of nuclear properties yet have very similar chemistry. The radiolanthanide, holmium-166 (^{166}Ho) is an attractive therapeutic radionuclide because of its short half-life (26.8 hr), deep penetration range (~9.0 mm in soft tissue), high beta energy with 1.77 MeV (48%) and 1.85 MeV (51%)

suitable for radiotherapy and a small portion of gamma rays with 0.08 MeV (6.6%) and 1.38 MeV (0.9%) suitable for imaging. [5,6,7].

A number of conjugation techniques have been developed for modification of biomolecules. The conjugation groups for attachment of a bifunctional chelating agent to biomolecules include anhydride, isothiocyanate, N-hydroxysuccinim- ide (NHS) ester, and maleimide. Anhydrides of diethylene triamine pentaacetic acid (DTPA) and tetraazacyclododecane tetraacetic acid (DOTA) have also been used to prepare their bioconjugates [18,19,20]. DTPA is an organic compound consisting of a diethylenetriamine backbone modified with five carboxymethyl groups, which has a high affinity for metal cations.

Technetium-99m (^{99m}Tc)-labeled Cetuximab has been applied to single photon emission computed tomography (SPECT) imaging for radioimmunodetection [14,15]. It has been reported that ^{99m}Tc-labeled Cetuximab has suitable dosimetric properties for a diagnostic nuclear medicine agent. The study with the long-lived positron emitter zirconium-89 (^{89}Zr) and other therapeutic radiometals (such as yttrium-90, and lutetium-177) were performed to predicting the biodistribution of other therapeutic radiometals (such as yttrium-90, and lutetium-177), when labeled to Cetuximab via different types of chelator (ie., *p*-SCN-Bz-DOTA, *p*-SCN-Bz-DTPA, etc.) [16]. Copper-64 (^{64}Cu)-DOTA-Cetuximab has also been utilized for EGFR imaging [17].

In this study, DTPA-Cetiximab was prepared and ^{166}Ho was labeled to the Cetuximab derivatives. ^{166}Ho-labeling conditions were optimized and the biological characteristics of the ^{166}Ho-labeled conjugate were examined in a EGFR-positive tumor model to evaluate the clinical applicability of ^{166}Ho-DTPA-Cetuximab as a therapeutic agent.

II. MATERIALS AND METHODS

1. Materials

Cetuximab was purchased from Merck Inc. (Germany). Diethylene triamine penta acetic acid anhydride (DTPA), Ethylene diamine tetra acetic acid (EDTA), ammonium acetate, acetic acid, sodium phosphate, and sodium bicarbonate were obtained from Sigma-aldrich Chemical Co. (USA). Casein blocker solution was purchased from BioRad (USA). Antibodies and conjugates were concentrated using Vivaspin-20 centrifugal filter devices (Sartorius, Germany) and a Allegra X-15R centrifuge (Beckman Coulter, USA). All solutions were made using distilled deionized water (Milli-Q; Millipore, Bedford, MA; 18 mΩ/cm resistivity). ^{166}Holmium was provided by Korea Atomic Energy Research Institute (KAERI, Korea). The radiochemical purity and radiolabelling yield were analyzed using Instant thin layer chromatography-silica gel (ITLC-SG, Pall Co., USA) and measured by a Thin-layer chromatogram scanner (Aloka, Japan). The radioactivity was counted on a 1480 WIZARD well-type gamma counter (PerkinElmer, USA). Gamma camera images were obtained with a clinical gamma camera (TRIAD TXLT-20, Trionix, USA).

2. Methods

2.1. Cell culture

Human lung adenocarcinoma A549, human epithelial carcinoma A431 and human glioma U87-MG cell were obtained from the American Type Culture Collection (Manassas, USA). Human colorectal adenocarcinoma SNU-C2A and CT-26 were obtained from Korean Cell Line Bank (Seoul, Korea). A431, U87-MG and CT-26 were maintained as monolayer cultures in DMEM (Gibco, USA), A549 and SNU-C2A cells in RPMI 1640 (Gibco, USA) supplemented with 10% fetal bovine serum (JR scientific Inc., Woodland, CA), 100 units/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37℃.

2.2. Evaluation of EGF receptor expression on the surface of cancer cells

2.2.1 *in vitro* cell binding assay

To evaluate EGFR expression levels in five different cancer cell lines, A549, A431, U87-MG, SNU-C2A and CT-26, *in vitro* cell binding assay was performed. All steps in the cell binding assay were performed at 4℃ except incubation at room temperature. The tumor cells were washed 3 times with phosphate-buffered saline (PBS, pH=7.4 without $CaCl_2$ and $MgCl_2$). Iodination

of Cetuximab was accomplished using Pierce® Pre-Coated Iodination Tubes (Pierce, USA). $Na^{125}I$ (100 μCi; PerkinElmer, Finland) and 50 mM sodium phosphate buffer (pH 7.5) were added in pre-coated tube up to 100 μl, which was then pre-incubated at room temperature for 15 min with vortexing at every 5 min. Cetuximab (100 μg/100 μl) was added in the $Na^{125}I$ solution and then incubated at room temperature for 30 min with vortexing at every 5 min. A radiolabeling yield was determined by instant thin-layer chromatography using a silica gel-coated sheet (ITLC-SG, Pall Co., USA) and acetone as a developing solution. One million cells were counted and incubated for 1 hr with ^{125}I-labeled Cetuximab (100 ng) in the presence or absence of an 100-fold molar excess of cold Cetuximab (10 μg). The cells were washed three times with a casein blocker solution (1%). Radioactivity of each sample was counted in a well-type gamma counter (1480 WIZARD, PerkinElmer, USA).

2.2.2 Reverse transcriptase-polymerase chain reaction (RT-PCR)

A549 and CT-26 cells (5×10^6 cells) were lysed and total RNA was extracted by Easy-spin™ total RNA preparation kit (Intron Biotechnology, Korea). cDNA was synthesized by Superscript kit (invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. RT-PCR of β-actin and epithermal growth factor receptor (EGFR) was performed in the presence of the primer oligonuclotides (Bionics, KOREA) (Table 1) [21]. The standard cycling program was as followed: 94 °C for 60 sec; 57 °C for 30 sec; and 72 °C for 90 sec. After 30 cycles of reaction, the PCR products were extended at 72 °C

for 7 min. After completion of the PCR, 10 μL aliquots of the reaction mixtures were analyzed on a 2% ethidium bromide-stained tris-acetate-EDTA (TAE) agarose gel.

Table 1. Primer sequences for β-actin and EGFR and amplified product sizes

Primer name	Sequence (5' → 3')	Product size (bp)
β-actin Forward	AGGCTGTGCTGTCCCTGTATGC	540
β-actin Reverse	ACCCAAGAAGGAAGGCTGGAAA	
EGFR Forward	GAGAGGAGAACTGCCAGAA	454
EGFR Reverse	GTAGCATTTATGGAGAGTG	

2.3. Experimental animal models

All animal studies were performed with the approval of the Korea Institute of Radiological and Medical Science Animal Care and Use Committee. Female athymic BALB/c-nu/nu (SLC, Japan), aged 5-6 weeks, were used for animal studies. For tumor implantation, A549 cells in log phase of growth were harvested with trypsin 0.25% and EDTA 0.02%. The cells were counted with a hemacytometer, the viability of the cells was confirmed with the trypan blue-dye exclusion test. Then, the cell number was adjusted to 5×10^6 cells/mL in the serum-free media. Each mouse was subcutaneously inoculated with 0.1 mL of the tumor suspension on the left thigh.

2.4. Preparation of DTPA-Cetuximab conjugates

To remove any metal ions in Cetuximab, Cetuximab solution (15 mg) was incubated for 30 min at room temperature with 75 μL of 0.1 M ammonium acetate buffer (pH 6) containing 50 mM EDTA and then concentrated with a centrifugal filter device (MW cut-off 50 K). The buffer of Cetuximab solution was changed to 0.25 M sodium bicarbonate (pH 8.3) and then concentrated to 1 mg/mL. Cyclic DTPA anhydride was conjugated to Cetuximab (10 mg) at molar ratios of 50:1, 100:1, 200:1, 300:1, 500:1 and 1000:1 (DTPA:Ab). The mixtures were incubated at 37℃ for 1 hr, and DTPA-Cetuximab conjugates were purified and concentrated by Vivaspin-20. The buffer was changed to 0.1 M ammonium acetate buffer. The final concentration of DTPA-Cetuximab was measured on UV absorbance at 280 nm.

2.5. ^{166}Ho-labeling to DTPA-Cetuximab

The procedure for the production of ^{166}Ho has been described in detail elsewhere [5]. Briefly, ^{166}Ho was produced at the HANARO (30 MW) research reactor of KAERI by the neutron irradiation of ^{165}Ho-nitrate [^{165}Ho (n, γ) ^{166}Ho]. Two hundred milligrams of $^{165}Ho(NO_3)_3 \cdot 5H_2O$ was weighed and sealed in a titanium capsule encased in an aluminum capsule and irradiated in the reactor for 3 days. The initial specific activity of Ho-166 [$^{166}Ho(NO_3)_3$] reached up to 11.7 GBq/mg (315.7 mCi/mg). After 24 hr cooling, the sample was dissolved in 3 mL of HCl solution (pH 3).

The DTPA-Cetuximab conjugates were labeled with ^{166}Ho, according to a previous report with minor modifications [22]. The average number of DTPA chelators per Cetuximab was determined using a previously reported procedure with minor modifications [23]. Briefly, a defined amount of nonradioactive $HoCl_3$ in 0.1 M ammonium acetate buffer (pH 6) was added to 0.4 mCi $^{166}HoCl_3$, and 100 μg of each DTPA-Cetuximab conjugate in 100 μL of 0.1 N ammonium acetate buffer were added to the above carrier-added $^{166}HoCl_3$ solution. The reaction mixture was incubated at room temperature for 30 min with vortexing at every 5 min. After purification of reaction mixture using Vivaspin-20, the number of DTPA molecules per Cetuximab was calculated as followed: Ho^{3+} moles×radiolabeling yield/DTPA-Cetuximab moles

^{166}Ho (13.88 MBq) was added to 1.5 mg of DTPA-Cetuximab conjugates synthesized at varied molar ratios of DTPA and Cetuximab. The reaction mixtures were incubated at room temperature for 30 min with vortexing at every 5 min. The radiolabeling yields and radiochemical purity of

^{166}Ho-DTPA-Cetuximab were assessed by ITLC-SG, developing with 0.15 M sodium acetate (pH 4.2). The plates were scanned and analyzed with a TLC scanner (Aloka, Japan).

2.6. Biochemical characterization of ^{166}Ho-DTPA-Cetuximab conjugates

2.6.1 Immunoreactivity assay of ^{166}Ho-DTPA-Cetuximab

To evaluate the immunoreactivity of ^{166}Ho-DTPA-Cetuximab, *in vitro* cell binding assay was performed with ^{166}Ho-DTPA-Cetuximab prepared at different molar ratio of DTPA and Cetuximab (50:1, 100:1, 200:1, 300:1, 500:1 and 1,000:1), and A549 cells (expressing EGFR). The relative immunoreactivity was calculated as (cell bound % of ^{166}Ho-DTPA-Cetuximab/cell bound % of ^{125}I-Cetuximab) × 100 (%)

2.6.2 *In vitro* Stability assay

In vitro stability of ^{166}Ho-DTPA-Cetuximab in serum proteins was examined for 24 hr. ^{166}Ho-DTPA-Cetuximab (200 µg/200 µL) was mixed with the same volume of human serum. The mixtures was then incubated in a 37℃ water bath for 1, 2, 5, 7, 18, and 24 hr. The stability of ^{166}Ho-DTPA-Cetuximab was assessed by radiochromatography with ITLC-SG as described earlier.

2.7. Biodistribution studies of ^{166}Ho-DTPA-Cetuximab conjugates in A549 tumor-bearing nude mice

Biodistribution analysis of ^{166}Ho-DTPA-Cetuximab conjugates was conducted in A549 tumor-bearing nude mice when the tumor size grew to a diameter of 1 cm at 4-5 weeks after tumor implantation. Each tumor-bearing mouse was injected with 0.1 mL of ^{166}Ho-DTPA-Cetuximab conjugate (460 KBq/50 µg) via tail vein. The mice (n=4 per each time point) were sacrificed at 2, 6, 24, 48, 72, and 144 hr after injection. The blood was collected by cardiac puncture and the organs of skin, muscle, femur, heart, lungs, liver, kidneys, spleen, stomach, small intestine, large intestine, and tumors were excised carefully. The organ samples were weighed and counted in a well-type gamma counter (1480 WIZARD well-type gamma counter, PerkinElmer, Finland). The accumulated radioactivity was expressed as a percentage of injected dose per gram of tissue (% ID/g). Tumor-to-blood (T/B) ratios and tumor-to-muscle (T/M) ratios were also calculated.

2.8. Gamma camera images of A549 tumor-bearing nude mice administered with ^{166}Ho-DTPA-Cetuximab conjugates

Gamma camera images of A549 tumor-bearing nude mice were obtained after intravenous administration of ^{166}Ho-DTPA-Cetuximab conjugates (580 KBq/100 μg) through a tail vein. At 24 hr postinjection, the animals were anesthetized by intraperitoneal injection of a mixture of ketamine (80 mg/Kg) and xylazine (12 mg/Kg). Tumor images were acquired in a gamma camera (TRIAD TXLT-20, Trionix, USA) at a 256 × 256 matrix (1×10^6 counts).

III. RESULTS

1. Evaluation of EGF receptor expression on the surface of cancer cells

In vitro Cetuximab cellular binding assay was performed to verify expression of EGF receptors in A431, A549, SNU-C2A, U87MG, and CT-26 cells. Cellular binding of Cetuximab (%) was calculated by a formula of (cell-bound radioactivity - non specific binding radioactivity)/total radioactivity × 100 (%). ^{125}I-Cetuximab was effectively bound to A431, A549, and SNU-C2A cells while little bound to the U87MG and CT-26 cells. Binding % of ^{125}I-Cetuximab to A431, A549, SNU-C2A, U87MG, and CT-26 cells was 29.0 ± 1.5, 33.0 ± 1.5, 27.8 ± 0.3, 0.3 ± 0.4 and 0.2 ± 0.3%, respectively (Figure 1). Presumably A549 cells have the highest expression of EGF receptors, resulting in the highest binding of Cetuximab to the cells.

cDNA was synthesized from the total RNA extracted from EGFR-positive A549 cells and EGFR-negative CT-26 cells. The cDNA sequences of EGFR were amplified with the designated primer oligonucleotides mentioned earlier (Table 1). According to the electrophoretic analysis of the amplified PCR product, the A549 cells express a high density of EGFR while the CT-26 cells do not (Figure 2). Based on these observations, the A549 cells were utilized as an EGFR-positive tumor model for further *in vitro* and *in vivo* studies in this research.

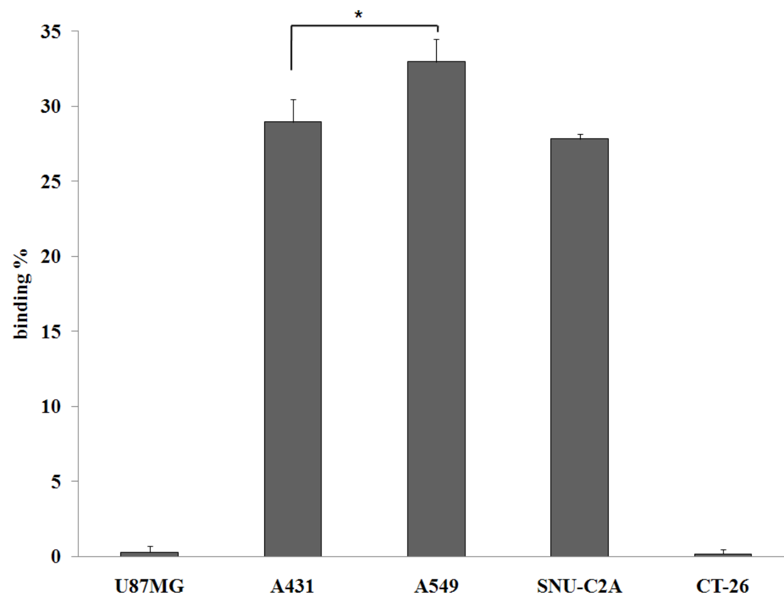

Figure 1. Immunoreactivity of Cetuximab to various types of cancer cells. A431, A549, SNU-C2A, U87MG, and CT-26 cells (each 1×10^6 cells) were incubated with ^{125}I-labeled Cetuximab for 1 hr at room temperature. After washing with a casein blocking solution three times, the radioactive antibodies bound to the cells were determined in a gamma counter as radioactivity % of added dose. Student's *t*-test of radioactivity in A431 cells vs. radioactivity in A549 cells, $p<0.05$.

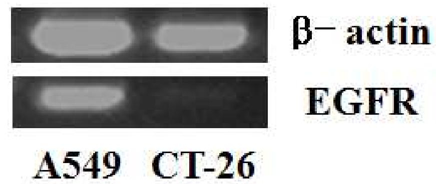

Figure 2. Verification of EGFR expression in the A549 and CT-26 tumor cells by RT-PCR. Total RNA of A549 tumor cells and CT-26 cells were extracted and the cDNA synthesized. RT-PCR of β-actin and EGFR were performed with appropriate primers (Table 1). After completion of the PCR, reaction mixtures were analyzed on a 2% ethidium bromide TAE agarose gel.

2. Preparation of DTPA-Cetuximab conjugate

Cetuximab was conjugated with cyclic DTPA anhydride at various ratios of DTPA to Cetuximab to find an optimal coupling condition. The conjugates were synthesized at DTPA/Cetuximab ratios of 50:1, 100:1, 200:1, 300:1, 500:1 and 1,000:1. The number of DTPA molecules per Cetuximab was calculated using the following equation; the number of DTPA per Cetuximab= Ho^{3+} moles×radiolabeling yield/DTPA-Cetuximab moles. The number of bound DTPA per Cetuximab was determined to be 22.0, 39.2, 49.2, 59.8, 73.2 and 88.4 at the DTPA/Cetuximab ratios mentioned above, respectively (Figure 3).

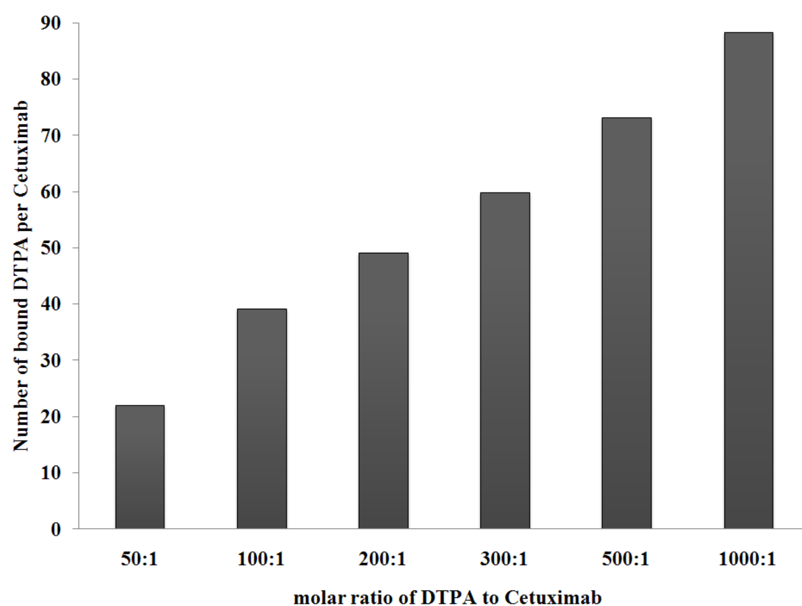

Figure 3. The number of DTPA molecules bound to Cetuximab depending on reacting their molar ratios. DTPA and Cetuximab were conjugated at varied their molar ratios. The amount of DTPA chelators bound to the Cetuximab was calculated by equation; the number of DTPA per Cetuximab= Ho^{3+} moles×radiolabeling yield/DTPA-Cetuximab moles.

3. Radiolabeling of ^{166}Ho to DTPA-Cetuximab and relative immunoreactivities of the labeled conjugates

When 0.15 M sodium acetate buffer (pH: 4.2) was used as a mobile phase, ^{166}Ho-DTPA-Cetuximab remained at the origin (R_f=0), and free ^{166}Ho migrated with the solvent front (R_f=1) (Figure 4). According to the ITLC-SG analysis, radiolabeling yields of ^{166}Ho to DTPA-Cetuximab conjugates prepared at 50:1, 100:1, 200:1, 300:1, 500:1 and 1000:1 molar ratios of DTPA to Cetuximab were 63.5, 69.4, 97.2, 98.2, 97.9, and 98.9%, respectively (Figure 5, Table 2). The specific activities of the ^{166}Ho-DTPA-Cetuximab conjugates were 23.3, 41.8, 52.2, 63.6, 77.7, and 94.0 MBq/mg, respectively as well (Table 2). Although the number of DTPA per Cetuximab was different among the six conjugates, their immunoreactivities to A549 cells were similar to each other, 48.2 ± 0.4, 58.6 ± 6.3, 60.6 ± 5.3, 54.8 ± 6.1, 59.2 ± 7.2, and 60.7 ± 3.0% at 50:1, 100:1, 200:1, 300:1, 500:1 and 1000:1 molar ratios of DTPA to Cetuximab (Figure 6). According to these data, the ^{166}Ho-DTPA-Cetuximab conjugates prepared at the 1000:1 molar ratio of DTPA to Cetuximab were utilized for further *in vitro* and *in vivo* experiments.

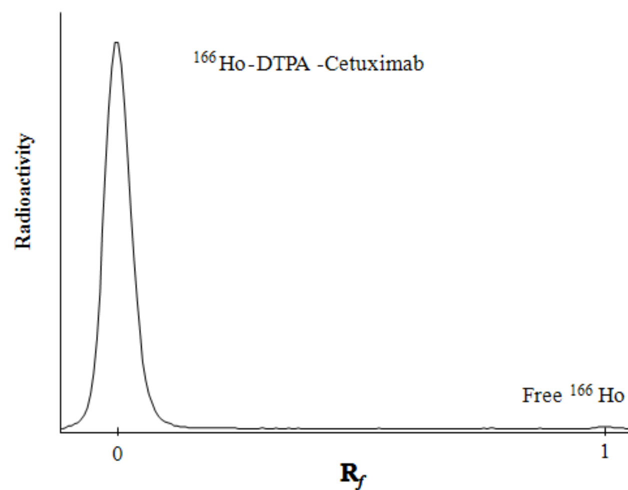

Figure 4. Radiochromatogram of ^{166}Ho-DTPA-Cetuximab. ITLC-SG as a stationary phase and 0.15 M sodium acetate buffer (pH: 4.2) as a mobile phase are used for radiochromatography. ^{166}Ho-DTPA-Cetuximab synthesized at the 1000:1 molar ratio of DTPA to Cetuximab remained at the origin (R_f=0), while free ^{166}Ho migrated with the solvent front (R_f=1).

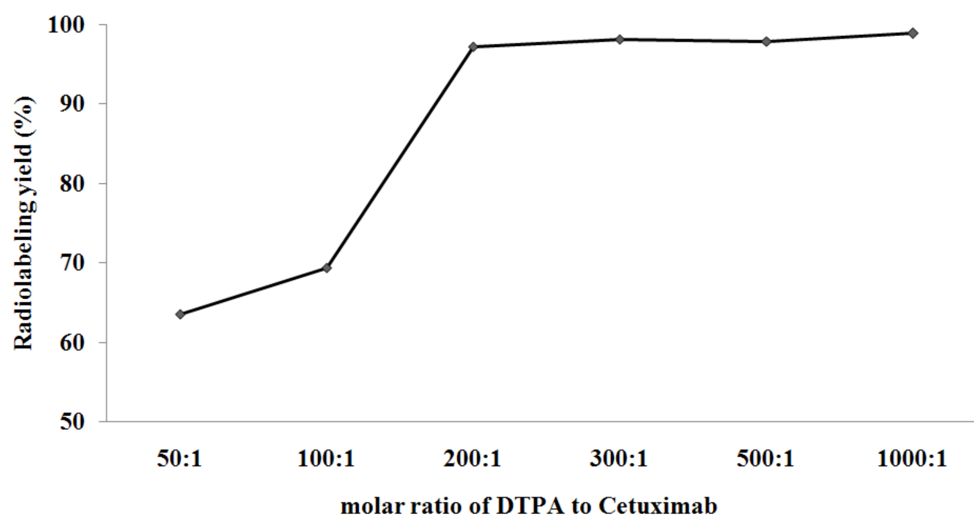

Figure 5. Radiolabeling yields of ^{166}Ho-DTPA-Cetuximab conjugates. ^{166}Ho was labeled to DTPA-Cetuximab conjugates synthesized at various molar ratios of DTPA to Cetuximab. Radiolabeling yields and radiochemical purity were assessed by ITLC-SG with the developing solvent of 0.15 M sodium acetate buffer solution (pH 4.2).

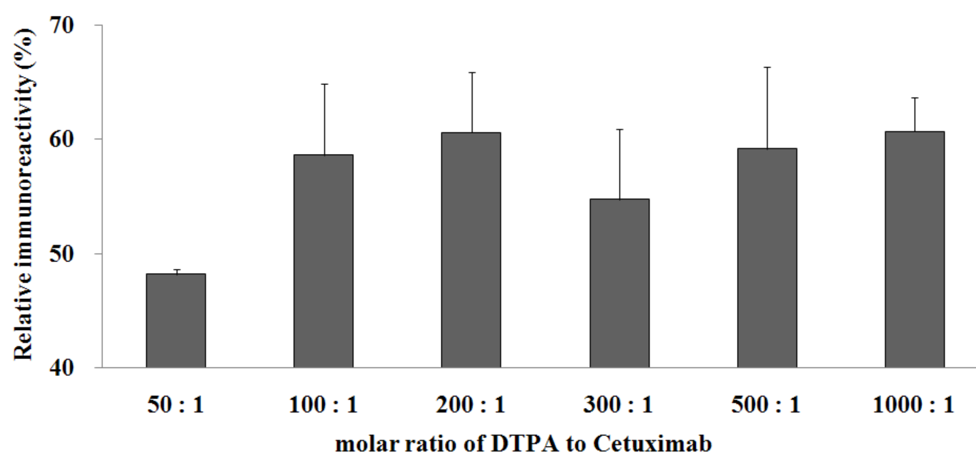

Figure 6. Immunoreactivity of ^{166}Ho-DTPA-Cetuximab to A549 cells. *In vitro* cellular binding assay was performed with ^{166}Ho-DTPA-Cetuximab synthesized at different molar ratios of DTPA to Cetuximab. Their immunoreactivities to A549 cells were calculated as cell binding % of ^{166}Ho-DTPA-Cetuximab/cell binding % of ^{125}I-Cetuximab × 100.

Table 2. The radiolabeling yield, number of DTPA per Cetuximab, specific activity, and relative immunoreactivity of ^{166}Ho-DTPA-Cetuximab conjugates prepared at varied ratios of DTPA and Cetuximab

DTPA/Cetuximab ratio	50:1	100:1	200:1	300:1	500:1	1000:1
Yield (%)	63.5	69.4	97.2	98.2	97.9	98.9
No. of DTPA per Ab	22.0	39.2	49.2	59.8	73.2	88.4
Specific activity (MBq/mg)	23.3	41.8	52.2	63.7	77.7	94.0
Relative Immunoreactivity (%)	48.2 ±0.4	58.6 ±6.3	60.6 ±5.3	54.8 ±6.1	59.2 ±7.2	60.7 ±3.0

4. *In vitro* stability of ^{166}Ho-DTPA-Cetuximab in serum

The stability of ^{166}Ho-DTPA-Cetuximab prepared at 1000:1 molar ratio of DTPA to Cetuximab was evaluated after incubation in the presence of serum proteins for 1, 2, 5, 7, 18 and 24 at 37℃. At the designated time points, intact ^{166}Ho-DTPA-Cetuximab conjugates were separated and analyzed by ITLC. Radioactivities of the ^{166}Ho-DTPA-Cetuximab conjugates were maintained 98 ± 0.5, 96 ± 0.5, 98 ± 0.3, 97 ± 0.9, 96 ± 1.8 and 95 ± 1.5% after incubation for 1, 2, 5, 7, 18 and 24 hr, respectively. According to these date, it can be concluded that the structure of ^{166}Ho-DTPA-Cetuximab conjugate is stable in serum proteins.

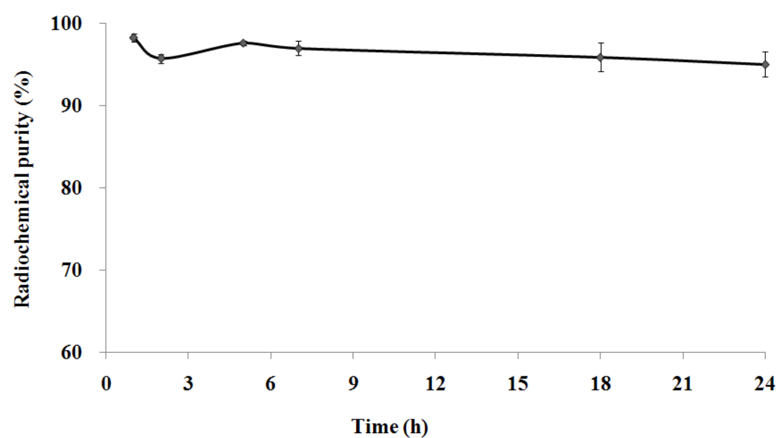

Figure 7. *In vitro* stability of ^{166}Ho-DTPA-Cetuximab in serum. ^{166}Ho-DTPA-Cetuximab was mixed with the same volume of human serum and then incubated in a 37℃ water bath for 1, 2, 5, 7, 18 and 24 hr. The stability of ^{166}Ho-DTPA-Cetuximab was assessed by ITLC-SG.

5. Biodistribution studies of ^{166}Ho-DTPA-Cetuximab conjugates in A549 tumor-bearing nude mice

The ^{166}Ho-DTPA-Cetuximab conjugates prepared at 1,000:1 molar ratio of DTPA to Cetuximab were injected to mice bearing A549 tumors and their biodistribution was analysed at varied time points. Intratumoral uptake of the ^{166}Ho-labeled Cetuximab was increased with time elapsed, 3.04 ± 1.36, 3.58 ± 1.90, 6.91 ± 0.34, 7.84 ± 1.74, 9.10 ± 1.83, and 7.95 ± 3.96% ID/g at 2, 6, 24, 48, 72, and 144 hr postinjection, respectively (Figure 8, Table 3). ^{166}Ho-DTPA-Cetuximab exhibited high uptake in the blood and liver at early time points, whereas the tracer uptake in the other organs except bones was a low level. Accumulation of ^{166}Ho-DTPA-Cetuximab in bones was also increased over time. The tumor/blood ratio of ^{166}Ho-DTPA-Cetuximab was only 0.12 ± 0.05% ID/g at 2 hr postinjection, but significantly increased to 9.09 ± 2.85% ID/g at 144 hr (Table 4). The tumor/muscle ratio of ^{166}Ho-DTPA-Cetuximab was also increased to 9.12 ± 3.68% ID/g at 144 hr postinjection. There data showed that ^{166}Ho-DTPA-Cetuximab was excreted via the urinary tract.

Table 3. Biodistribution of ^{166}Ho-DTPA-Cetuximab conjugates in A549 tumor-bearing mice

Unit : %ID/g *

Organ	2hr	6hr	24hr	48hr	72hr	144hr
Blood	24.1±2.1	17.9±1.0	8.3±1.6	4.4±0.3	4.2±2.3	0.9±0.3
Liver	12.2±0.7	12.3±0.9	16.6±2.4	17.6±2.3	11.6±2.2	8.0±2.0
Lung	8.8±0.7	6.3±1.0	3.9±0.9	3.0±0.3	3.2±0.6	1.5±0.3
Spleen	5.3±0.7	4.6±0.9	6.3±1.4	9.7±3.2	6.8±1.0	6.4±1.5
Kidney	9.2±1.0	7.1±0.3	5.1±0.5	3.6±0.5	3.1±0.8	1.5±0.3
Stomach	0.9±0.3	0.7±0.4	1.9±0.2	0.5±0.4	0.7±0.1	0.6±0.3
S. Intestine	3.2±0.3	2.2±0.2	2.1±0.7	1.1±0.3	0.8±0.2	0.4±0.1
L. Intestine	2.9±0.2	2.2±0.2	2.5±0.5	1.5±0.8	1.3±0.2	0.4±0.1
Thyroid	4.3±0.5	3.7±0.4	4.0±1.3	2.4±0.3	3.8±0.5	3.2±0.4
Femur	3.6±1.0	5.0±0.1	6.1±2.1	6.3±1.3	9.0±2.3	8.9±0.8
Muscle	2.1±0.6	2.0±0.2	2.8±1.1	1.0±0.1	1.5±0.3	1.1±0.8
A549	3.0±1.4	3.6±1.9	6.9±0.3	7.8±1.7	9.1±1.8	8.0±4.0

* Values at each time point represent the mean ±s.d. of percentage of injected dose per gram of tissue weight (n=4).

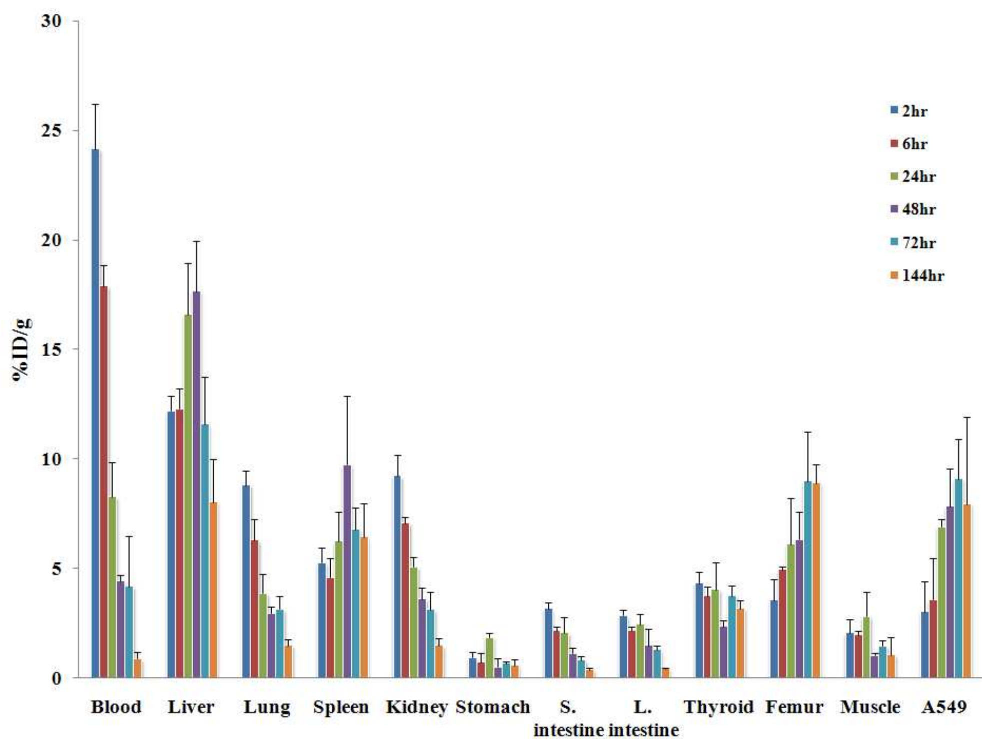

Figure 8. Biodistribution of ^{166}Ho-DTPA-Cetuximab conjugates in A549 tumor-bearing mice. Each tumor-bearing mouse was injected with 0.1 mL of ^{166}Ho-DTPA-Cetuximab conjugates (460 KBq/50 μg) into a tail vein. Collected organs were weighed and radioactivities of the organs were counted in a well-type gamma counter. The accumulated radioactivity was expressed as a percentage of injected dose per gram of tissue (% ID/g).

Table 4. Organ per Tissue ratio of ^{166}Ho-DTPA-Cetuximab conjugates in A549 tumor-bearing mice

Organ/Tissue	2hr	6hr	24hr	48hr	72hr	144hr
Tumor/Blood ratio	0.1±0.1 *	0.2±0.1	0.9±0.2	1.8±0.3	2.6±1.1	9.1±2.9
Tumor/Muscle ratio	1.7±1.2	1.8±1.0	2.7±2.4	7.8±2.4	6.2±0.9	9.1±3.7

* Mean ± S.D., n=4.

6. Gamma camera images of A549 tumor-bearing nude mice administered with ^{166}Ho-DTPA-Cetuximab conjugate

Gamma camera images of A549 tumor-bearing nude mice were acquired to evaluate *in vivo* tumor targetability of ^{166}Ho-DTPA-Cetuximab. ^{166}Ho-DTPA-Cetuximab conjugates (580 KBq/100 μg) were administrated through a tail vein. At 24 hr postinjection, tumor images were acquired in a 256 × 256 matrix (1×10^6 counts). According to the gamma camera image at 24 hr postinjection, ^{166}Ho-DTPA-Cetuximab was selectively localized in the left thigh with EGFR-positive A549 tumors, but not in the control right thigh. However, the resolution of the gamma images was not good enough to provide clear selective localization of the ^{166}Ho-DTPA-Cetuximab in the A549 xenograft.

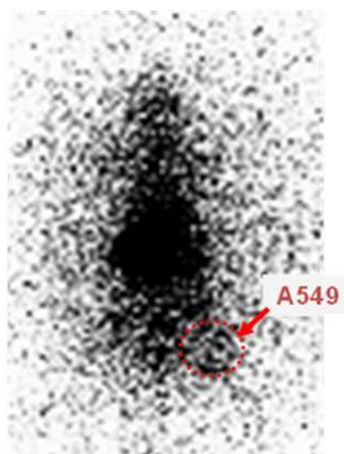

Figure 9. A gamma camera image of A549 tumor-bearing nude mouse administered with ^{166}Ho-DTPA-Cetuximab conjugates. Gamma camera images of A549 tumor-bearing nude mouse treated with ^{166}Ho-DTPA-Cetuximab conjugates were obtained after intravenous injection of ^{166}Ho-DTPA-Cetuximab conjugates (580 KBq/ 100 μg). At 24 hr postinjection, the mouse was anesthetized by intraperitoneal injection of a mixture of ketamine (80 mg/Kg) and xylazine (12 mg/Kg). Tumor images were acquired in a 256 × 256 matrix (1×10^6 counts).

IV. DISCUSSION

Many radioisotopes and monoclonal antibodies have been widely used in the diagnosis and therapy of various types of cancers. Especially, in recent targeted RIT has been clinically validated and approved for the treatment of cancer by the US Food and Drug Administration.

To label ^{166}Ho to Cetuximab, first of all chelators for radioisotope, DTPA in this study, have to be conjugated to Cetuximab molecules. Therefore, conjugation conditions have to be optimized to have the stable DTPA-Cetuximab conjugates with a strong immunoreactivity against tumor cells. DTPA is known to stably chelate metals such as ^{99m}Tc [24]. The DTPA was added to the Cetuximab solution at the DTPA/Cetuximab ratios of 50:1, 100:1, 200:1, 300:1, 500:1 and 1,000:1 used for conjugation reaction. The higher amount of DTPA was added, the higher number of DTPA was coupled to Cetuximab molecules. At the 1,000:1 ratio, approximately 88 DTPA molecules were conjugated to one molecule of Cetuximab antibody. ^{166}Ho-DTPA-Cetuximab conjugated at the 1,000:1 ratio exhibited the highest radiolabeling yield and the highest specific activity among the conjugates.

Also, the relative immunoreactivity of the ^{68}Ho-labeled Cetuximab was increased with increase of DTPA number per molecule. Previously it has been reported that conjugation of bifunctional chelating agents diminishes immunoreactivity of monoclonal antibodies [25]. However, in this study, the immunoreactivity of Cetuximab was little affected by the increased number of DTPA. This suggests that the accessible lysine residues for DTPA conjugation are fairly away from the complementarity-determining region of Cetuximab. In

addition, the ^{166}Ho-DTPA-Cetuximab was very stable in serum proteins. The radiochemical purity of ^{166}Ho-DTPA-Cetuximab was reduced only less than 5% after incubation with human serum at 37℃ for 24 hr.

The immunoreactivity of the ^{68}Ho-labeled Cetuximab was examined in the A549 tumor model. In order to establish an appropriate tumor model for *in vitro* and *in vivo* experiments, A431, A549, SNU-C2A, U87MG, and CT-26 tumor cells were treated with ^{125}I-labeled Cetuximab. ^{125}I is a convenient radionuclide for laboratory studies because it has relatively long half-life (60.14 d) and emits a short-range of Auger and Coster-Kronig electrons [26]. According to the immunoreactivity assay with the cancer cell lines, A549 cells showed the highest affinity to the ^{125}I-labeled Cetuximab than the other types of tumor cells, implying the highest expression of EGF receptors. The EGFR expression in A549 cells was also confirmed by RT-PCR.

The ^{166}Ho-DTPA-Cetuximab prepared at the 1,000:1 molar ratio of DTPA to Cetuximab was able to effectively accumulated in the A549 tumor xenografts *in vivo*. The intratumoral uptake of ^{166}Ho-DTPA-Cetuximab was increased with time elapsed and reached a maximum at 72 hr postinjection. The ^{166}Ho-labeded Cetuximab exhibited a relatively higher uptake in the blood, liver and bones at early time points. It is important to note that free ^{68}Ga and ^{111}In tend to localize in the liver and lungs due to their strong binding capability to transferrin while ^{90}Y and lanthanide isotopes are readily deposited on the bone [27]. The tumor/blood ratio and tumor/muscle ratio of ^{166}Ho-DTPA-Cetuximab reached 9.1 ± 2.9% ID/g and 9.1 ± 3.7% ID/g at 144 hr postinjection. This result also shows the specific localization of the conjugates in A549 tumors mediated by Cetuximab.

However, the low resolution of gamma camera images which were acquired after intravenous injection of ^{166}Ho-DTPA-Cetuximab was rather disappointing. ^{166}Ho-DTPA-Cetuximab appeared to be selectively localized in the A549 tumor xenograft at 24 hr postinjection, but gave a poor resolution of image. These results suggest that ^{166}Ho-DTPA-Cetuximab need a further optimization process before preclinical and clinical applications as a radioimmunotherapeutic agent against EGFR expressing tumors.

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국문 요약

^{166}Ho-DTPA-Cetuximab의 제조와 생물학적 평가

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Ho의 방사성동위원소 ^{166}Ho(γ에너지: 0.081 MeV, 1.38 MeV, β^-에너지: 1.78 MeV, 1.84 MeV, 반감기: 26.8 hr)은 β^-의 큰 에너지를 방출하기 때문에 방사면역치료에 적합한 물리적 특성을 지니고 있으며 γ선에 의한 핵의학 영상이 가능한 장점이 있다. Cetuximab은 EGFR에 대한 키메릭 단클론 항체로서 현재 EGFR을 과발현하는 고형암 치료에 사용되고 있다. 본 연구에서는 ^{166}Ho을 먼저 bifunctional chelating agent인 DTPA와 conjugation 시킨 후 Cetuximab에 방사성 표지하여 종양형성 누드마우스에서 생체 분포 및 생체 영상을 평가함으로써 방사면역진단 및 치료제로써 이용가능성을 보고자 하였다. EGFR의 발현 정도를 평가하기 위해서 5가지 종양세포(A431, A549, SNU-C2A, U87MG, CT-26 tumor cells)에서 ^{125}I-Cetuximab을 이용해 *in vitro* cell binding assay를 실시하였다. A549 종양세포가 다른 나머지 세포들에 비해 높은 EGFR의 발현을 한다는 것을 확인한 후 RT-PCR을 통해 A549 세포의 EGFR의 발현을 확인하였다. Cetuximab에 다양한 몰비 (50:1,

100:1, 200:1, 300:1, 500:1, 1000:1)의 cyclic DTPA를 반응시켜 DTPA-Cetuximab을 제조하였다. Cetuximab에 결합한 DTPA의 수는 DTPA와 Cetuximab의 몰비가 1000:1일 때 88.4개로 가장 많은 수가 결합하였고 ^{166}Ho의 방사성 표지수율 또한 98.9%로 가장 높았다. ^{166}Ho-DTPA-Cetuximab의 면역반응성과 비방사능은 각각 60.7 ± 3.0%와 94.0 MBq/mg이었다. A549 종양이식 마우스에서 ^{166}Ho-DTPA-Cetuximab은 72시간째에 가장 높은 종양으로의 집적(9.1 ± 1.8% ID/g)을 나타냈고 144시간째에 가장 높은 종양 대 혈액비 (9.1 ± 2.9)와 종양 대 근육비 (9.1 ± 3.7)를 나타내었다. 또한 ^{166}Ho-DTPA-Cetuximab을 A549 종양 형성 누드마우스에 주사하여 24 시간 후에 gamma camera 영상에서 종양으로의 집적을 확인할 수 있었다. 이 결과들을 통해 ^{166}Ho을 방사성 표지한 높은 비방사능을 가진 DTPA-Cetuximab이 EGFR을 과다 발현하는 종양에 대한 방사면역치료제로의 이용가능성을 확인하였다.

핵심어 : Cetuximab, ^{166}Ho, DTPA, 방사면역치료