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In vivo fluorescence imaging and single photon emission computed tomography using a novel human monoclonal antibody against Claudin-3 in ovarian cancer

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2023 년 02 월

서울대학교 대학원
의과학과 의과학전공
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In vivo fluorescence imaging and single photon emission computed tomography using a novel human monoclonal antibody against Claudin-3 in ovarian cancer

by

Sera Oh

A thesis submitted to the Department of Biomedical Sciences in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Biomedical Sciences at Seoul National University College of Medicine

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논문 제목: Visualization of a novel human monoclonal antibody against Claudin-3 for targeting ovarian cancer

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서울대학교총장 귀하

Abstract

In vivo fluorescence imaging and single photon emission computed tomography using a novel human monoclonal antibody against Claudin-3 in ovarian cancer

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Introduction: : Claudin-3 (CLDN3), a tight junction protein, regulates cell-to-cell interactions in epithelial or endothelial cell sheets. During tumorigenesis, epithelial cells are transformed, and tumor cells proliferate through out-of-plane division, resulting in external exposure of CLDN3. Since alterations of CLDN3 expression are associated with cancer progression and higher CLDN3 expression is observed in most

ovarian cancers, In this study, the feasibility of using a CLDN3-specific antibody was evaluated as a novel imaging tracer.

Methods: : After reducing the CLDN3-specific antibodies to expose the -SH groups, click chemistry was used to conjugate the radioactive isotope [^{111}In] or the fluorescent protein FNR648. Human ovarian cancer OVCAR-3 and glioblastoma U87MG cells were used as CLDN3-positive and -negative cells. Flow cytometry was used to determine the CLDN3 IgG1 monoclonal antibody binding to both cell lines. OVCAR-3 cells were injected subcutaneously into mice to establish a xenograft model. ^{111}In-labeled CLDN3 antibodies (370 kBq/50 μL) were administered intravenously into mice. After 24 h, organs, including tumors, were excised and measured with a γ–counter. Images were acquired with the IVIS optical imaging system and SPECT/CT.

Results: The labeling efficiency of [^{111}In]In-NOTA and [^{111}In]In-NOTA-antibody was 98.52% and 100%, respectively. FNR648-labeled CLDN3 antibody bound to the cell surface of OVCAR-3 and U87MG with 83.4%

and 5.7% specificity, respectively. In OVCAR-3 tumor xenografted mice, CLDN3 IgG1 antibody showed a 2.5-fold higher tumor uptake (20.4 ± 7.4% ID/g) than control IgG1 (8.8 ± 2.6% ID/g) at 24 h post injection. The CLDN3 antibody fluorescence signal in the tumor peaked at 24 h post injection.

Conclusion: In this study, CLDN3-specific antibody was successfully conjugated with a radioisotope and a fluorescent protein and was verified the specific binding of labeled antibodies to OVCAR-3 tumors in a mouse model. These data suggest that CLDN3-specific human monoclonal antibodies could be used as a useful theranostic tracer.

Keywords : Claudin-3, Human monoclonal antibody, Dual imaging, Imaging tracer, Human ovarian cancer

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LIST OF ABBREVIATIONS

ADCC, Antibody-dependent cellular cytotoxicity

BCA, Bicinchoninic acid

BLI, Bioluminescence imaging

CLDN3, Claudin-3

CPE, Clostridium perfringens enterotoxin

CT, Computed tomography

DBCO, Dibenzocyclooctyne

ECL, Extracellular loop

FACS, Fluorescence activated cell sorting

ITLC-SG, Instant Thin Layer Chromatography-Silica Gel

N_3-NOTA, 3-azidopropyl-1,4,7-triaza-cyclononane-1,4,7-triacetic acid

scFv, Single-chain variable fragment

SPECT, Single photon emission computed tomography

TJ, Tight junction

%ID, Percent injected dose

INTRODUCTION

Carcinoma, a malignant tumor originating from epithelial tissues, accounts for 80 ~ 90% of all human cancers. In normal cells, junctions between adjacent epithelial cells form polarity by blocking the movement of various proteins and lipids that are critical to normal cell function. The most important feature of advanced tumors is the loss of polarity in epithelial cells [1]. The tight junction (TJ), which controls permeability to epithelial cell sheets and acts as a barrier to intramembrane diffusion [2], is involved in tumorigenesis and metastasis. Malignant cells often exhibit abnormal tight junctions and cell polarity [3]. Reduced cell-cell interactions due to TJ loss lead to disruption of the polarity of epithelial sheets, resulting in malignant morphological features [4]. Thus, TJ components play an important role in the development and progression of tumors and are potential cancer targets [5].

Claudins (CLDNs) are major transmembrane proteins that form the

TJ backbone and are required for its assembly, barrier, and paracellular permeability functions [5]. CLDNs consist of a 24-member gene family and are expressed in epithelial and endothelial cells, maintaining TJ cell polarity and preventing diffusion of membrane proteins from the apical to the basolateral cell membrane [6]. CLDN3 (220 amino acids) consists of four transmembrane domains with cytoplasmic N- and C-termini and two extracellular loops, ECL1 (27-80th amino acids) and ECL2 (144-159th amino acids) [7]. Although the role of CLDNs in tumorigenesis is not yet clear, high expression of CLDN3 has been reported to correlate with progression and recurrence in a variety of cancers, including breast, colorectal, gastric, pancreatic, ovarian, and prostate [8-11]. In particular, high expression of CLDN3 is known to correlate with poor prognosis and survival [8, 12-15]. Many studies have recently investigated CLDN3 as a therapeutic target using overexpression of CLDN3 in various carcinomas [14-18].

Although CLDN3 plays a pivotal role in tight junction integrity, recent studies have shown that it is also independently involved in cancer

cell survival and invasion. CLDN3 has been proposed as a therapeutic target as it is overexpressed in various carcinomas, particularly in aggressive metastatic tumors. Clostridium perfringens enterotoxin (CPE), which causes food poisoning, recognizes ECL2 of CLDN3 and CLDN4 [12, 19], enabling the development of CPE-based therapeutics in various cancers with increased expression of CLDN3 or CLDN4 [19]. Therefore, there is a need to develop drugs with high specificity, low immunogenicity, and low cytotoxicity.

Therapeutic antibodies specifically target antigens overexpressed in tumor cells and induce cell death in tumors through various mechanisms [20, 21]. Many therapeutic antibodies have been developed, and more than 80 have been approved by the United States Food and Drug Administration (FDA) [22]. Therapeutic antibodies kill tumor cells either directly or through immune-mediated mechanisms of antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) [23, 24]. Although therapeutic antibodies have been successfully used as a monotherapy, they have been further developed to

enhance therapeutic efficacy through more specific and selective targeting involving cytotoxic agents, radioactive isotopes, toxins, or photosensitizers [25-28]. Antibody-based targeted imaging enables non-invasive whole-body imaging and has been used in clinical practice for early cancer diagnosis, detection of cancer lesions, and prognostic monitoring of treatment [29].

In a previous study, a human immunoglobulin G1 (IgG1) monoclonal antibodies against CLDN3 were successfully produced from a human single-chain variable fragment (scFv) phage library using CLDN3-expressing CHO-K1 cells and CLDN3-containing lipoparticles [30, 31]. In this study, the feasibility of using a CLDN3-specific antibody was evaluated as a novel imaging tracer.

MATERIALS AND METHODS

1. *In vitro* study

1.1. Cell lines and culture

Human ovarian cancer (OVCAR-3), breast cancer (MCF7), and glioblastoma (U87MG) cells were used to test CLDN3 expression and establish xenograft mouse models. OVCAR-3 cells were cultured in RPMI-1640 (Welgene Inc., Gyeongsan-si, Korea) with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% antibiotics (Invitrogen, Waltham, MA, USA). MCF7 and U87MG cells were maintained in MEM (Minimum essential medium, Welgene Inc., Gyeongsan-si, Korea) with 10% FBS and 1% antibiotics. Cells were grown in a humidified atmosphere at 37℃ containing 5% CO_2.

1.2. Evaluation of CLDN3 protein expression in various human cancer cell lines

Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0 with 150 mM sodium chloride, 1% Igepal CA-630, 0.5% sodium deoxycholate

and 0.1% sodium dodecyl sulfate), and cell lysates were centrifuged at 13,000 rpm for 10 min. Protein concentration was quantified by the BCA protein assay kit (ThermoFisher, Waltham, MA, USA). 10 μg of protein from each cell line was separated on a 15% SDS-PAGE gel and transferred to a polyvinyl difluoride membrane (PVDF, Millipore, Watford, UK). After blocking the PVDF membranes with 5% skim milk in tris-buffered saline for 1 h at room temperature, the membranes were incubated overnight at 4°C with primary antibodies to CLDN3 (Abcam, Cambridge, UK; diluted 1:1000) or β-actin (Sigma Aldrich, St. Louis, MO, USA; diluted 1:5000). The membranes were subsequently probed with HRP-conjugated anti-rabbit or anti-mouse IgG (Cell Signaling Technology, Danvers, MA, USA; diluted 1:1000). Visualization was performed using ECL reagents (Roche, Basel, Switzerland). Signal intensity was measured using a chemiluminescence imaging system (Bio-Rad, Hercules, CA, USA).

1.3. Flow cytometric analysis to evaluate CLDN3 expression at the cellular level in human cancer cell lines

To confirm CLDN3 expression, 5×10^5 cancer cells were incubated in PBS containing 1% FBS with FNR648-conjugated control IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA) or CLDN3-specific antibodies [30, 31] (5 μg/mL) for 30 min on ice. Cells were then washed 3 times with PBS containing 1% FBS. Stained cells were analyzed with a BD Accuri C6 Plus FACS system (BD Biosciences, Franklin lakes, NJ ,USA).

1.4. Immunofluorescence staining for visualization of CLDN3 expression in human cancer cell lines

Cells were seeded on cover glass in each well of a 12-well plate and cultured for 24 h. Subsequently, cells were fixed with 4% paraformaldehyde for 30 min, washed with PBS, and blocked with 5% BSA in PBS for 30 min. Fixed cells were incubated with the anti-CLDN3 antibody (Abcam, Cambridge, UK; diluted 1:100) at RT for 1 h, and then incubated with Alexa-flour 488-conjugated anti-rabbit antibody (Invitrogen, Waltham, MA, USA; diluted 1:800) for 30 min. Nuclear staining and mounting were performed with Prolong gold with DAPI

(Invitrogen, Waltham, MA, USA). After fixing and drying at -20°C, fluorescence images were obtained with a confocal microscope (Carl Zeiss, Jena, Germany).

1.5. Radioisotope or fluorescence labeling of CLDN3-specific antibody for *in vivo* dual imaging

To expose thiol groups of the antibody, 1 mM TCEP was added and incubated with shaking at 37°C for 2 h. For conjugation with radioisotopes or fluorophore, 2 μL of 20 nmol/μL of DBCO-PEG4-maleimide (Dibenzocyclooctyne-PEG4-maleimide, 1 μmole/μL, dissolved in DMSO) was added and reacted at room temperature for 2 h. The ratio of the CLDN3-specific antibody IgG (Abion, Seoul, Korea) to ADIBO was used in a 1:10 reaction for binding with the –SH groups. Conjugated antibodies were purified using a PD-10 column and eluted with PBS at each step.

The pH of [^{111}In]$InCl_3$ was adjusted to pH 5 using 1 M sodium acetate buffer (NaOAc, pH 5) and 3-azidopropyl-NOTA (3-azidopropyl-

1,4,7-triaza-cyclononane-1,4,7-triacetic acid, 18 nmol dissolved in 10 μL DMSO) was added to pH-adjusted [^{111}In]$InCl_3$. The reaction mixture was incubated at 70°C for 10 min in a heating block. Subsequently, [^{111}In]In-3-azidopropyl was added to DBCO-CLDN3 antibodies at 37°C for 1 h in a heating block. At each step, the labeling efficiency of products (3-azidopropyl-NOTA with [^{111}In] or 3-azidopropyl-^{111}In with the DBCO-CLDN3 antibodies) was determined using Instant Thin Layer Chromatography (ITLC-SG) with 0.1 M citric acid as a solvent. Strips were counted with a Bio-Scan AR-2000 System Imaging Scanner (Bio-Scan Inc., Washington D.C., USA).

For fluorophore conjugation, 10 nmole/μL of 3-azidopropyl-FNR648 (Flamma® fluors 648) (BioActs, Incheon, Korea) was added to CLDN3-specific antibodies, and reacted at room temperature for 1 h, and fluorescence-labeled antibodies were purified using the PD-10 column and eluted with PBS.

1.6. Serum stability test of ^{111}In-labeled CLDN3 antibody

Human serum was filtered using a Minisart syringe filter (hydrophilic, 0.2 μm, Sartorius Stedim Biotech., Bohemia, NY, USA) and 5 mL syringe (BD, Franklin Lakes, NJ, USA). Filtered human serum was mixed with [^{111}In] labeled CLDN3-specific antibodies and incubated at 37°C in a shaking incubator. At each time point (0, 4, 12, 24, 48, 72, and 96 h after mixing with radiolabeled antibodies and human serum), the mixture was analyzed using ITLC-SG with 0.1 M citric acid as the solvent. Strips were counted with a Bio-Scan AR-2000 System imaging scanner (Bio-Scan Inc., Washington D.C., USA).

1.7. *In vitro* cellular uptake of ^{111}In-labeled anti-CLDN3

Cells were seeded in a 6-well plate until they reached approximately 80% confluency. Next, the cells were trypsinized and 1×10^6 cells were added to a 5-mL test tube containing wamed Hank's balanced salt solution. After ^{111}In-labeled anti-CLDN3 mAb of 0.074 mBq (2 μCi/mL) was added to the cells, the tubes were incubated in a shaking incubator for 1 h at RT. These cells were then washed three times with cold Hank's

balanced salt solution and lysed for 5 min in 1% sodium dodecyl sulfate. Cell lysates were collected and radioactivity was measured using a Cobra II gamma counter (Canberra Packard, Ontario, Canada). Radioactivity was normalized to the amount of total protein at the time of the assay. Total protein levels were quantified using the BCA protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's recommendations and bovine serum albumin was used as a protein standard. All experiments were performed in triplicate.

2. *In vivo* study

2.1. CLDN3 targeted *in vivo* imaging using radioisotope or fluorescently conjugated antibody and biodistribution analysis

OVCAR-3 cells (5×10^6 cells) in 100 μL PBS were injected subcutaneously into the right flank of a 6-week-old female athymic nude mouse (Orient Bio, Seongnam, Gyeonggi, Korea) to generate xenografts. FNR648-labeled antibodies (65 μg/100 μL in PBS) were intravenously

administrated to the tumor-bearing mice. After 0, 4, 24, and 48 h of injection, mice were anesthetized with isoflurane and FNR648-labeled antibodies were visualized using an IVIS imaging system (Perkin Elmer, Waltham, MA, USA). Fluorescence was detected using excitation (645 nm) and emission (680 nm) filters.

After administration of ^{111}In-labeled CLDN3 antibody (11.47 MBq ± 0.523) to OVCAR-3 tumor-bearing mice by tail vein injection, SPECT/CT images were acquired at 0, 4, 24 and 48 h post injection using nanoSPECT/CT plus (Mediso, Budapest, Hungary). The scanning acquisition parameters for the imaging modality are 245 keV ± 10% γ-ray energy window. SPECT scans were acquired with 40 projections at an 18° angular step. CT images were acquired with a tube voltage of 45 kVp, an exposure time of 1.5 sections per projection, and a reconstruction algorithm of cone-beam filtered back-projection was used. Images were processed using InVivoScope (BioScan, Washington D.C., USA) software and a Gaussian reconstruction filter was used for SPECT images. After sacrificing the mice, the heart, lung, liver, kidney, spleen, stomach, intestine, and tumor were excised. Organs from each mouse

were placed in a 100 mm petri dish and imaged using IVIS 100 (Perkin Elmer, Waltham, MA, USA).

For biodistribution experiments, ^{111}In-labeled CLDN3 antibody (370 kBq/50 μL) was intravenously injected into tumor-bearing mice. After 24 h, mice were sacrificed and organs including tumors were excised. Tissues of interest were weighed and radioactivity was measured with a γ-counter (Canberra Packard, Ontario, Canada). For blocking experiments, mice were co-injected with unlabeled CLDN3 antibody (3 mg).

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (19-0128-S1A3).

2.2. Statistical analysis

Statistical comparisons were made using unpaired Student's t-test or one-way ANOVA. Data are presented as mean ± standard deviation (SD) of the mean for n = 3 to 4 replicates and the significance of the

values is indicated by asterisks (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$). All statistical analysis was measured using GraphPad Prism 8 software (San Diego, CA).

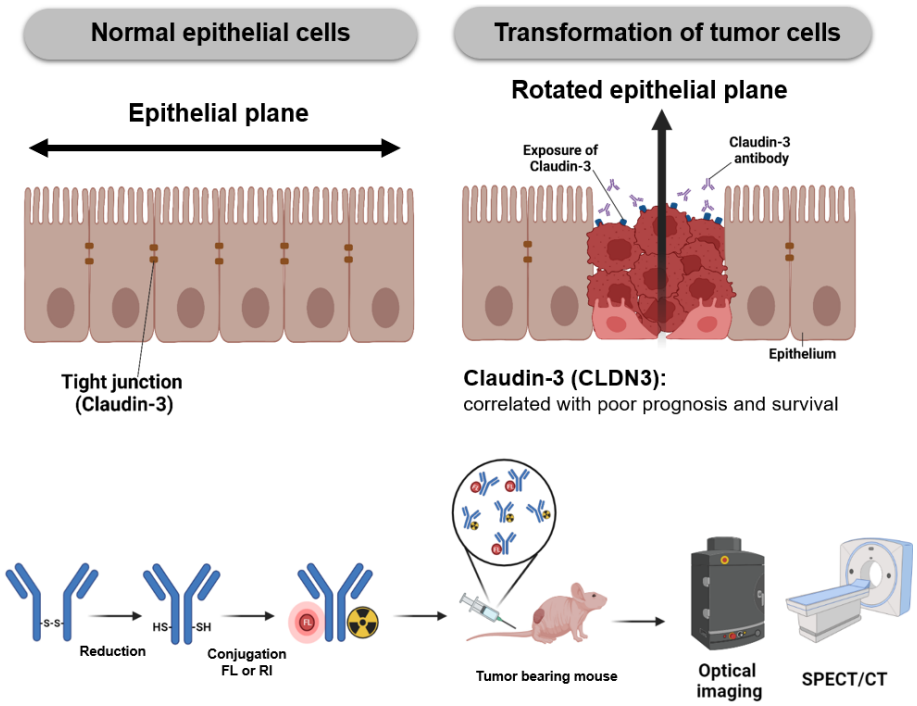


Figure 1. Schematic experiment of labeling CLDN3-specific antibody with radioisotopes or fluorophore

Among proteins forming tight junctions, CLDN3 plays a role in maintaining parallel structures in normal endothelial cells and regulates intercellular interactions. During tumorigenesis, out-of-plane division occurs, exposing CLDN3 to the outside. In addition, it is known that the expression of CLDN3 is closely related to poor prognosis and survival, and CLDN3 is overexpressed in most epithelial ovarian cancers. The

purpose of this study is to evaluate an image tracer that can simultaneously use optical or nuclear medicine imaging using a CLDN3-specific antibody. The labeled CLDN3-specific antibody was injected into ovarian cancer mouse model and evaluated as a dual image tracer that can be used for cancer diagnosis by optical or nuclear medical imaging.

RESULTS

1. Evaluation of CLDN3 expression and selection of CLDN3-positive cells

First, CLDN3 expression was characterized in various human cancer cell lines, including glioblastoma cell line U87MG, breast cancer cell line MCF7, and ovarian cancer cell line OVCAR-3. Western blotting showed high expression of CLDN3 protein in MCF7 and OVCAR-3 cell lines but no expression in U87MG (Figure 2a).

Cells were analyzed by flow cytometry to confirm the efficacy of the CLDN3-specific antibodies. Similar to Western blotting results, 79% and 83.4% of antibody binding was observed in MCF7 and OVCAR-3, respectively, indicating high expression of CLDN3. On the other hand, U87MG with low CLDN3 expression showed a binding affinity of 5.7% (Figure 3d). Immunofluorescence images of CLDN3 expression showed positive signals in the cell membrane of the CLDN3-expressing cell lines. No signal was observed in the CLDN3-negative cell line, U87MG, indicating the absence of non-specific binding (Figure 4a). As a result of

calculating and quantifying the fluorescence intensity from the immunofluorescence images, the fluorescence intensity of Alexa 488-conjugated CLDN3 antibody was the highest in MCF7 cell lines and showed a statistically significant difference from the other two cell lines, U87MG and OVCAR-3 (Fig 4b). Thus, the expression of CLDN3 was confirmed in human cancer cells, and the OVCAR-3 cell line was used as CLDN3-positive to target CLDN3.

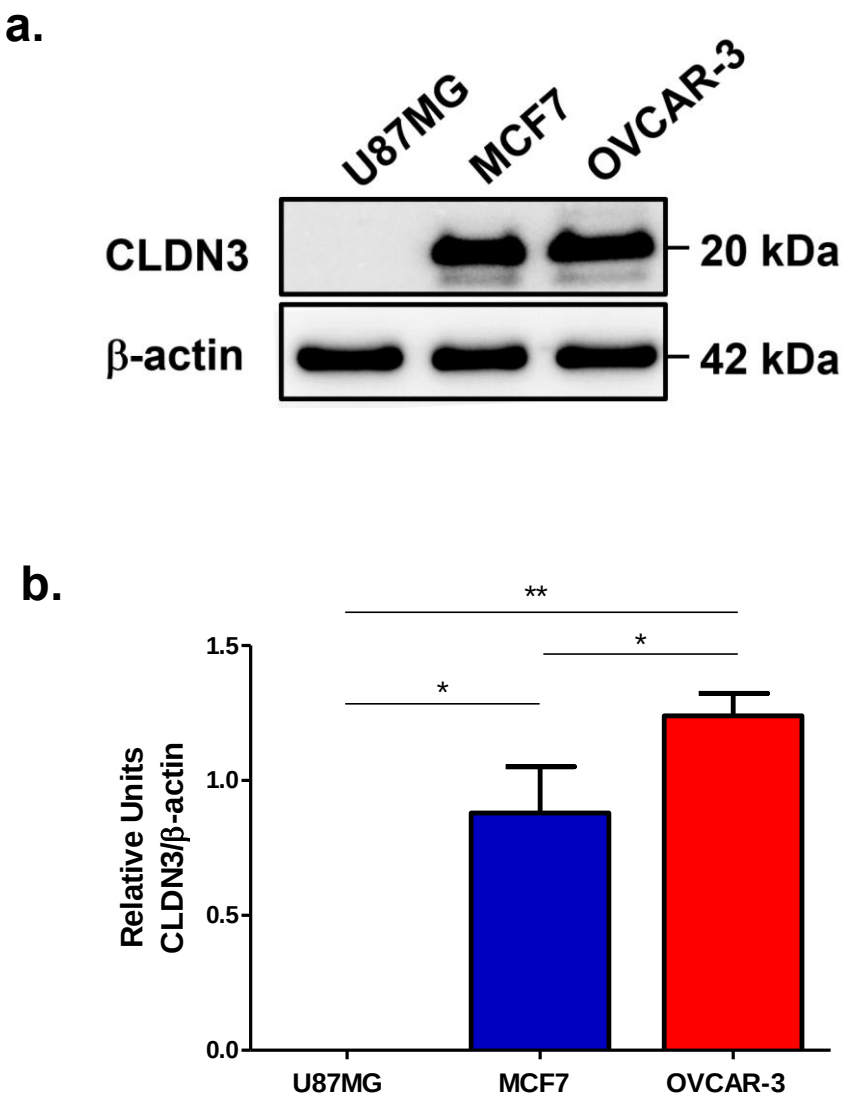


Figure 2. Expression of CLDN3 in various human cancer cell lines

(a) Western blot analysis of CLDN3 protein expression in various human cancer cell lines, negative cell line U87MG (human glioblastoma),

positive cell line MCF7 (human breast cancer cell line), OVCAR-3 (human ovarian cancer cell line). (b) CLDN3 protein expression level related by β-actin. N.D: Not detectable.

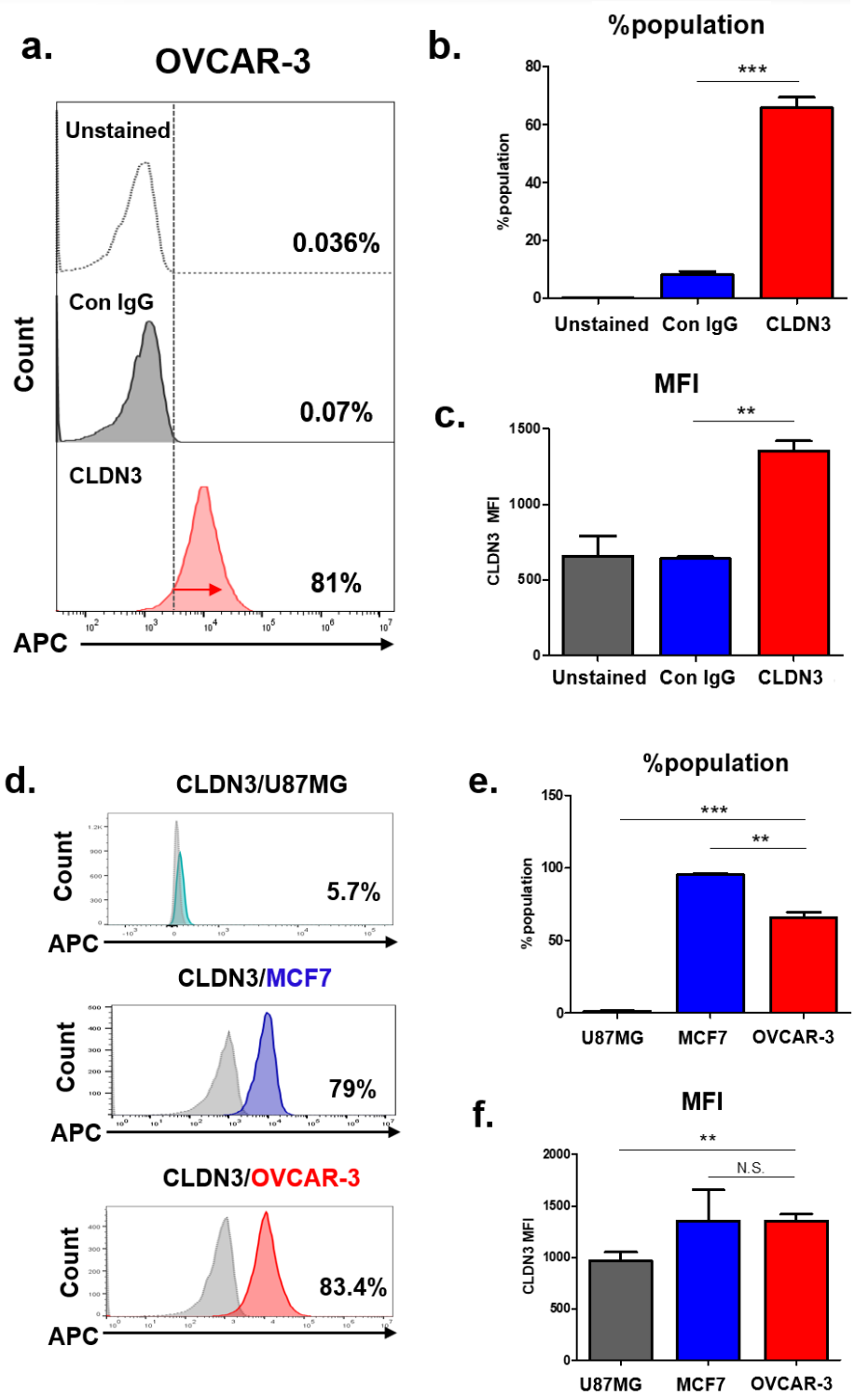
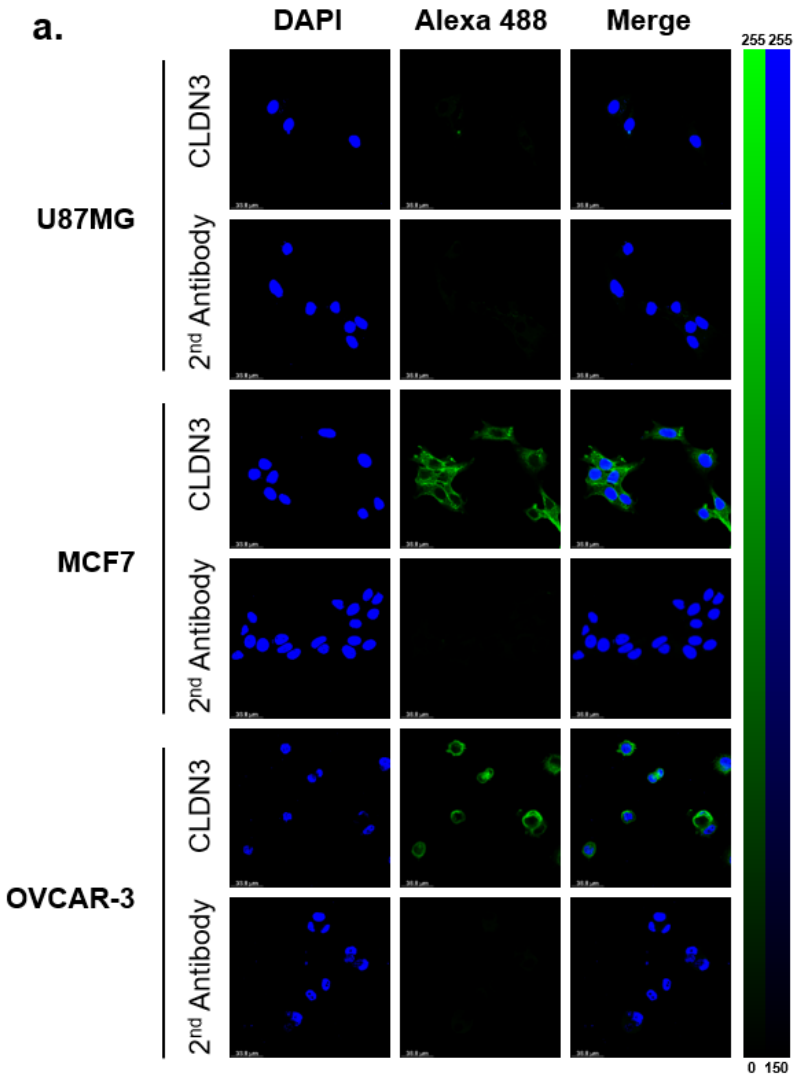


Figure 3. Expression of CLDN3 in various human cancer cell lines

Analysis of binding affinity of fluorescently conjugated CLDN3 in CLDN3 overexpressed cell lines. (a) Compared to con IgG, the CLDN3-specific antibody showed 81% binding affinity to the OVCAR-3 cell line. (b) As a result of analyzing the CLDN3 bound cell population using the FNR648-labeled CLDN3 antibody, the CLDN3 antibody was bound over 80% in OVCAR-3 cell line. (c) Cells treated with FNR648-CLDN3 antibody showed much stronger fluorescence intensity and a much higher MFI values than other groups. (d) Flow cytometric analysis of CLDN3 antibody bound in various human cancer cell lines. (e) In the CLDN3 overexpressed cell lines, the CLDN3-antibody bound was 79% in the MCF7 (human brease cancer cell line) and 83.4% in the OVCAR-3 (human ovarian cancer cell line), respectively. (f) In particular, the fluorescence bound CLDN3 antibody was the strongest in the MCF7 cell line and showed a higher MFI than other groups.


a.
DAPI
Alexa 488
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MCF7
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CLDN3
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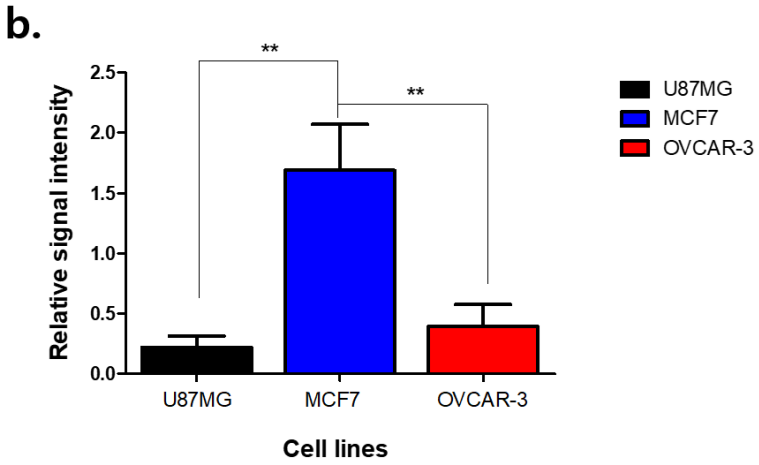
b.
Relative signal intensity
2.5
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U87MG
MCF7
OVCAR-3
Cell lines

Figure 4. Confirmation of CLDN3 expression by immunofluorescence staining in various human cell lines

U87MG, MCF7 and OVCAR-3 cells were fixed with 4% paraformaldehyde, incubated with anti-CLDN3 for 1 h at RT, and stained with Alexa 488 conjugated anti-rabbit antibody for 30 min. (a) Fluorescence was observed by confocal microscopy. (b) Quantitative values of cells stained using Alexa 488 conjugated CLDN3 antibodies were calculated and graphed.

2. Characterization of labeled CLDN3-specific antibody

Human monoclonal CLDN3-specific antibodies [30, 31]. were modified to expose thiol groups and labeled with ^{111}In using click reaction (Figure 5). According to TLC results, the radiochemical purity of [^{111}In]In-NOTA-N_3 was more than 98%, and the purity of ^{111}In labeled Con IgG (Figure 6a) and CLDN3-specific antibodies was 100% (Figure 6b). The stability of the labeled antibodies was over 80% at 37°C for up to 96 h in PBS and human serum (Figure 6c).

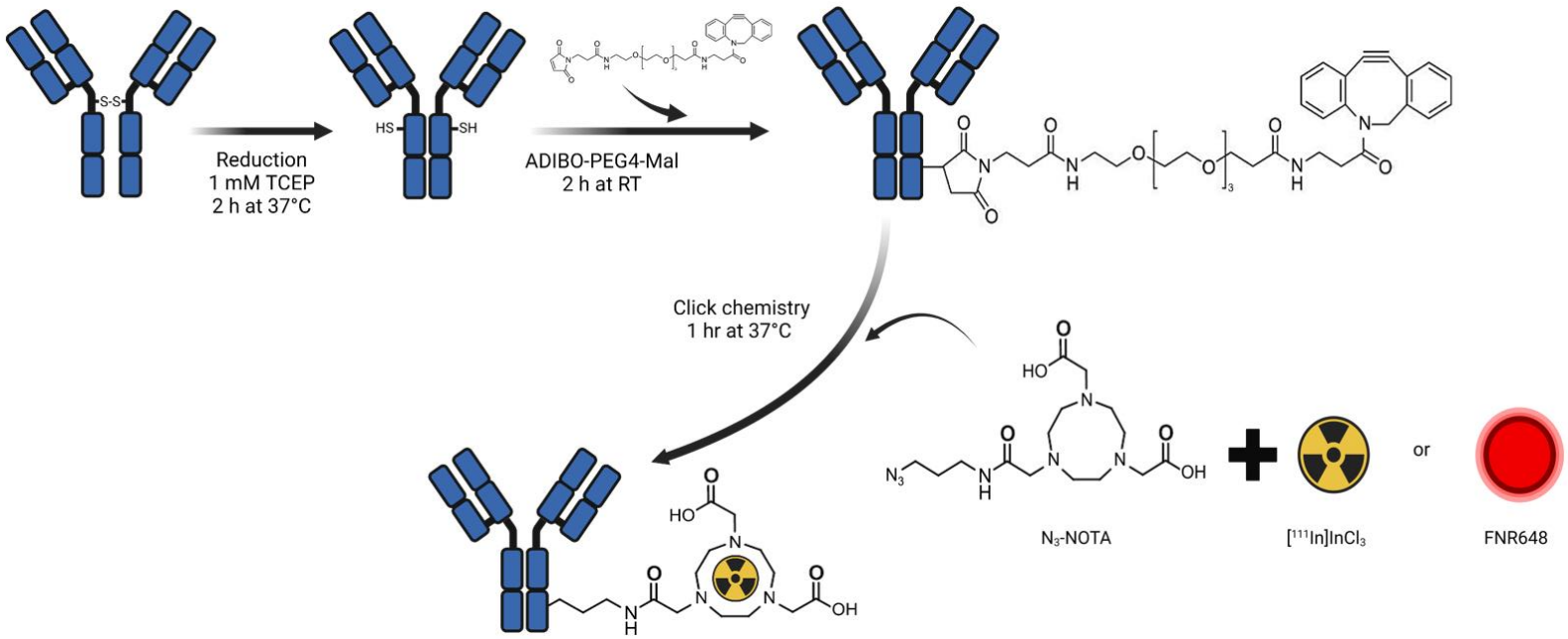


Figure 5. Preparation of labeling CLDN3-specific antibody with radioisotopes

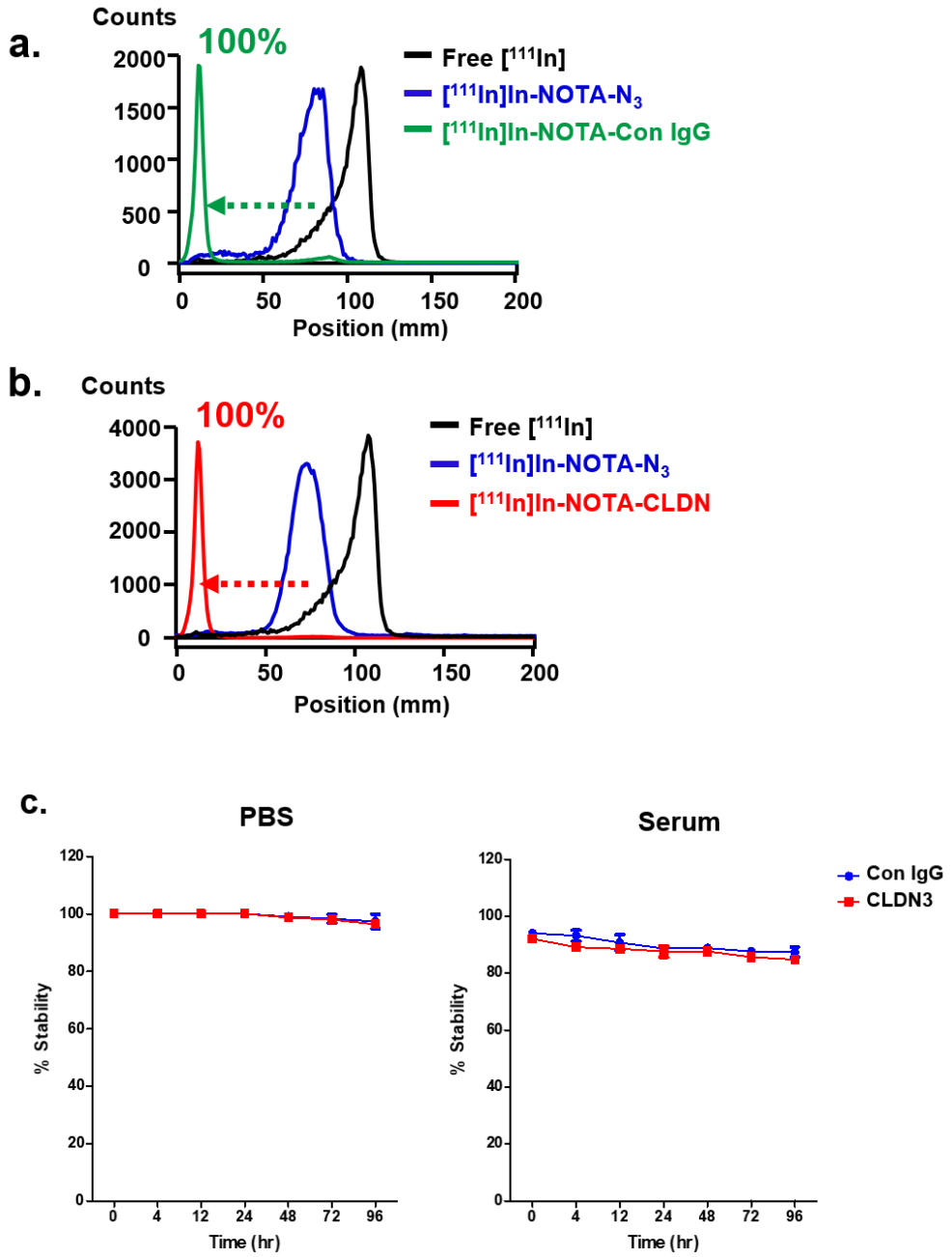


Figure 6. Evaluation of the activity of human monoclonal antibody for CLDN3 *in vitro*

(a) Radio thin-layer chromatography (TLC) analysis for the confirmation of ^{111}In-labeled Con IgG antibody, (b) ^{111}In-labeled CLDN3 antibody. ^{111}In was successfully chelated to NOTA-N_3 with a labeling efficiency of over 98%. According to the TLC results, both Con IgG and CLDN3 antibodies showed 100% labeling efficiency. (c) In vitro antibody stability in human serum. To evaluate the stability in human serum, each labeled antibody was added to human serum and measured up to 0, 4, 12, 24, 48, 72, and 96 h. Each antibody was maintained in a shaking incubator at 37°C. It was shown that the stability of the antibody in PBS was more than 90% and was stable up to 96 h. Similarly, ^{111}In-labeled-CLDN3 antibody in human serum was stable at more than 80% up to 96 h.

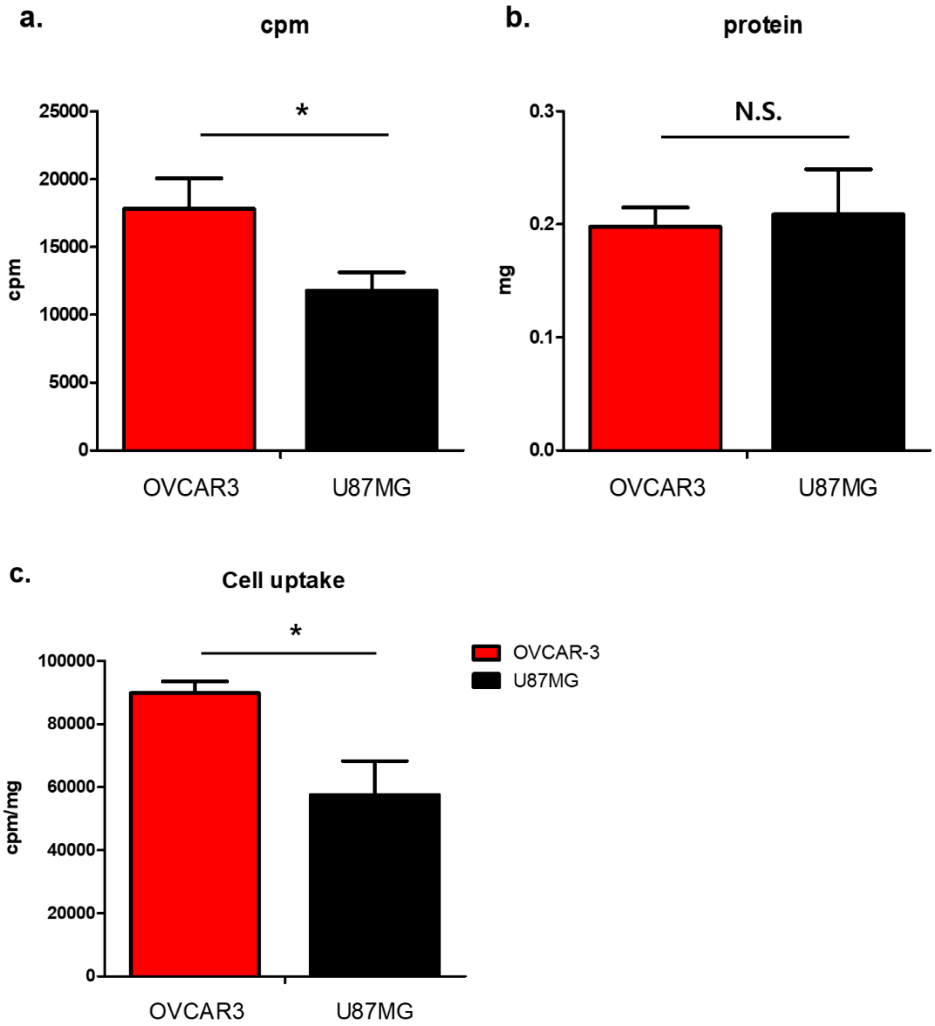


Figure 7. *In vitro* binding affinity of ^{111}In-labeled CLDN3 antibody

All ^{111}In-labeled-anti-CLDN3 cellular binding affinity assays in cells were measured by radioactivity of ^{111}In (γ-emitter) using a gamma counter. (a) CLDN3 overexpressed cell line, OVCAR-3, showed higher radioacitity values. On the other hand, U87MG with lower CLDN3 expression showed lower radioactivity, which showed a statistically

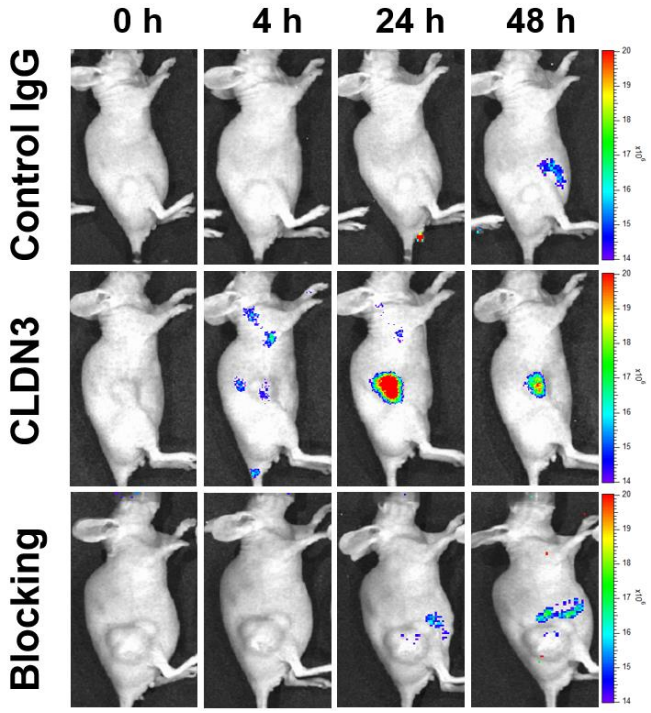
significant. (b) As a result of quantifying protein isolated from each cell lines for normalization, there was no statistically significant difference in the amount of protein in the two cell lines. (c) As a result of normalization, OVCAR-3 showed higher radioacitivity, indicating that ^{111}In-labeled-CLDN3 antibody more specifically bound to OVCAR-3.

3. Fluorescence images of CLDN3-specific antibody in a mouse model

Mouse tumor models were established using CLDN3-expressing OVCAR-3 cells and whether CLDN3-specific antibodies were recognized in vivo was investigated. FNR648-conjugated CLDN3 antibodies and control human IgG1 (con IgG) were injected intravenously into tumor-bearing mice and biodistribution images were compared in a time-dependent manner. Con IgG had no fluorescence signal in the tumor, but the CLDN3 antibody showed very specific fluorescence signal. To confirm the non-specific binding of the CLDN3 antibody, 3 mg of the non-labeled antibody and the FNR648-labeled CLDN3 antibody were intravenously injected into the mouse. Fluorescence was almost invisible in normal tissues, and non-labeled antibodies successfully blocked FNR648-labeled CLDN3 antibody binding in the tumor. (Figure 8a). As a result of quantification for each mouse, it was remarkably accumulated at the tumor site in mice injected with CLDN3 specific antibody and it was higher than that of the control group and the blocking group (Figure 8b). At 24 h post injection of the

antibody, the fluorescence intensity in mice injected with the CLDN3-specific antibody increased up to 10-fold compared to other groups, followed by gradual clearance over time (Table 1). Quantitative analysis of the fluorescence tumor images revealed specific binding of FNR648-labeled CLDN3 antibody in the tumor (Figure 8). Fluorescence measurement of ex vivo organs also demonstrated specific binding of FNR648-labeled CLDN3 antibody in the tumor (Figure 9). Taken together, the ability of fluorescently labeled CLDN3-specific antibodies to target CLDN3-expressing tumors in vivo was demonstrated, suggesting that fluorescently labeled antibodies have the potential to be used as diagnostic imaging tracers for tumor detection.

a.


0 h
4 h
24 h
48 h
Control IgG
CLDN3
Blocking

b.

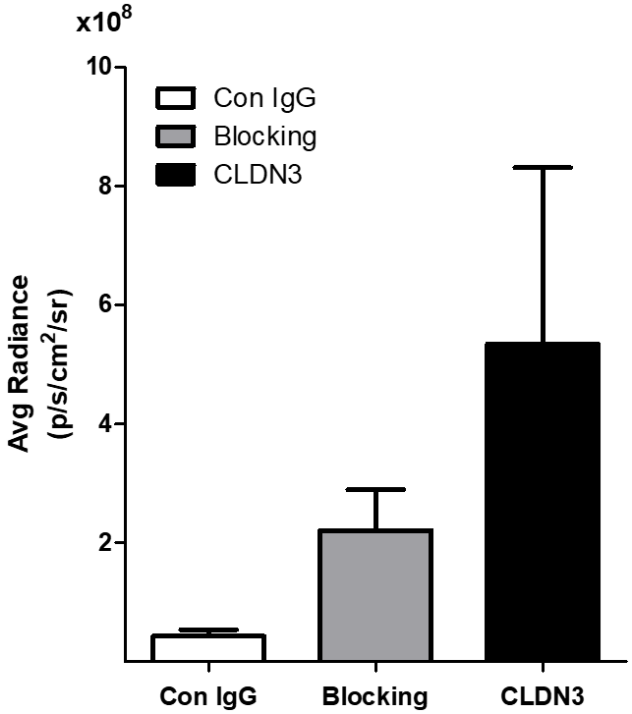

x10^8
10
8
6
4
2
Avg Radiance
(p/s/cm²/sr)
Con IgG
Blocking
CLDN3
Con IgG
Blocking
CLDN3

Figure 8. Fluorscence imaging of control IgG and CLDN3 specific antibody

(a) Fluorescence imaging of control IgG and CLDN3 specific antibody in OVCAR-3 tumor bearing mouse. OVCAR-3 cells were transplanted subcutaneously into athymic nude mice. Control human IgG and CLDN3 were intravenously injected at a dose of 100 μg/mouse, and the fluorescence intensity was monitored at 0, 4, 24, 48 h using IVIS100. (b) Mean fluorescence intensity by group. In the case of the blocking group, 3 mg of non-labeled antibody was pre-injected before injecting the fluorescently conjugated antibody. There was almost no fluorescence intensity until the first 4 h. At 24 h after injection, a strong fluorescence signal was detected at the tumor site in the mouse injected with the CLDN3 antibody. After that, the fluorescence intensity decreased over time. (b) As a result of the fluorescence intensity of the tumor region of each mouse at 24 h post-injection, the mice injected with CLDN3 showed much stronger fluorescence intensity than the other groups. In addition, the fluorescence intensity showed the highest peak at 24 h post-injection.

Table 1. Quantified fluorescence intensity of FNR648-labeled CLDN3 antibody

p/s/cm^2/sr	0 hr	4 hr	24 hr	48 hr
Control	7.16E+06	7.72E+06	8.10E+06	8.26E+06
CLDN3	4.44E+07	7.18E+07	8.57E+07	6.29E+07
Blocking	8.45E+06	8.96E+06	1.08E+07	1.15E+07

This table shows the fluorescence intensity at the tumor site time-dependent-manner. The fluorescence intensity was remarkably accumulated in the tumor site of the mice injected with the CLDN3-specific antibody. After 24 h of antibody injection, mice injected with CLDN3 specific antibody showed the highest fluorescence intensity, which was increased up to 10-fold compared to other groups.

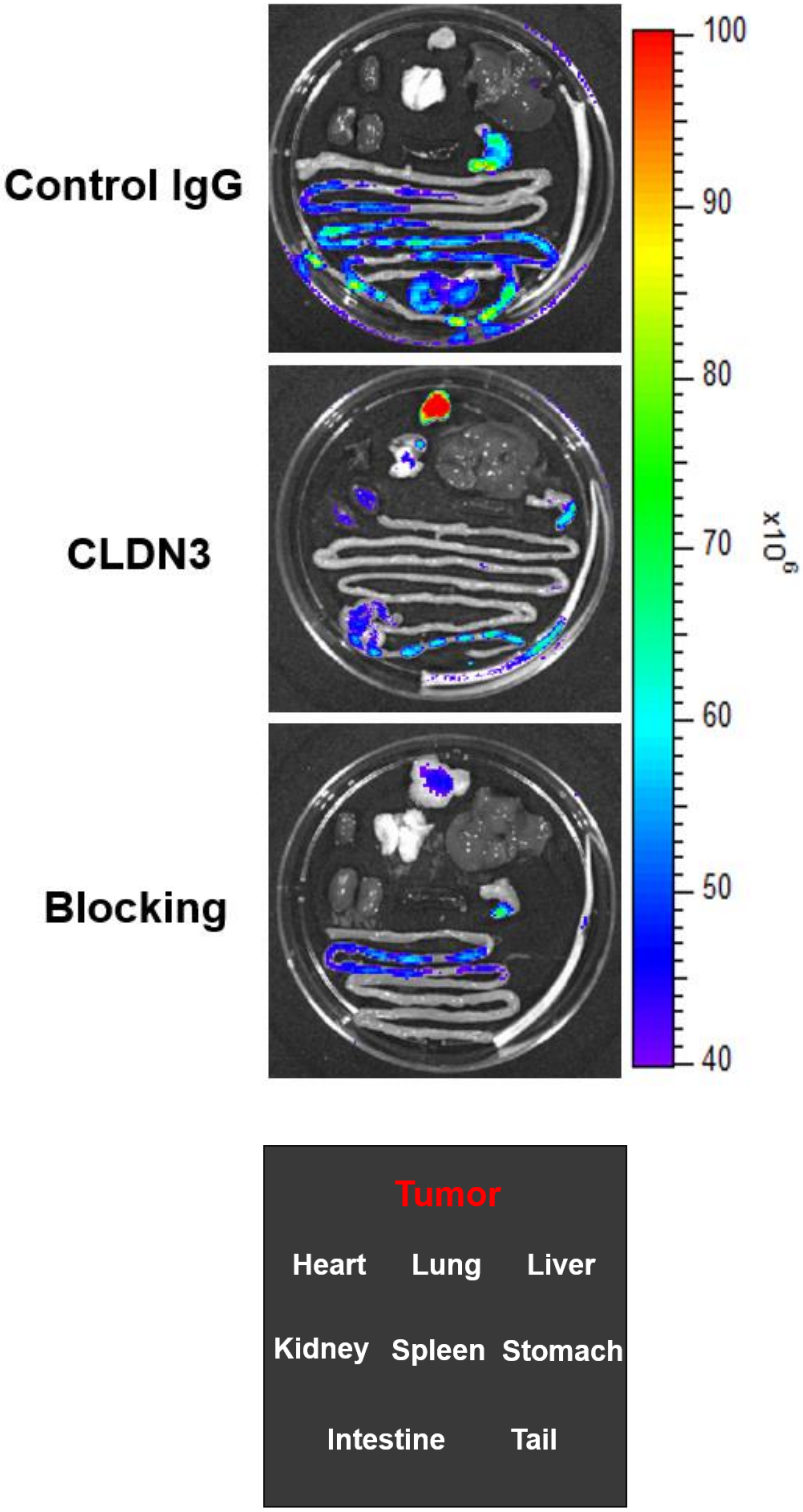

Control IgG
CLDN3
Blocking
100
90
80
70
60
50
40
x10^6
Tumor
Heart
Lung
Liver
Kidney
Spleen
Stomach
Intestine
Tail

Figure 9. Ex vivo fluorescence imaging

Ex vivo images of FNR648 conjugated CLDN3 antibody at 48 h post injection. As a result of fluorescence measurement in ex vivo organs, it was shown that the FNR648-labeled CLDN3-specific antibody effectively targeted CLDN3-expressing tumors and 7showed higher fluorescence intensity than other organs.

4. SPECT/CT imaging of the CLDN3-specific antibody in a mouse model

CLDN3 specific antibodies were labeled with ^{111}In for SPECT images to test the possibility of clinical application. SPECT/CT images were obtained by intravenous injection of ^{111}In-labeled antibodies. Uptake of ^{111}In-labeled control IgG was not observed in the tumor, and it was gradually removed from other organs over time. As expected, the ^{111}In-labeled CLDN3-specific antibodies showed significant uptake in OVCAR-3 tumors, with the highest tumor uptake observed at 24 h post-injection. On the other hand, mice injected with non-labeled antibodies for blocking showed lower uptake in the tumor and other organs (Figure 10). In summary, ^{111}In-labeled CLDN3 antibody exhibited significantly higher and specific tumor uptake in the OVCAR-3 tumor model. Biodistribution study also demonstrated higher uptake of ^{111}In-labeled CLDN3 antibody in tumors (20.6 ± 7.5 %ID/g vs 8.1 ± 3.6 %ID/g, $P < 0.05$) than that in the blocking group (Figure 11). When the distribution of each organ was compared, tumor uptake was the most prominent, but kidney uptake was also seen to some extent (Table 2).

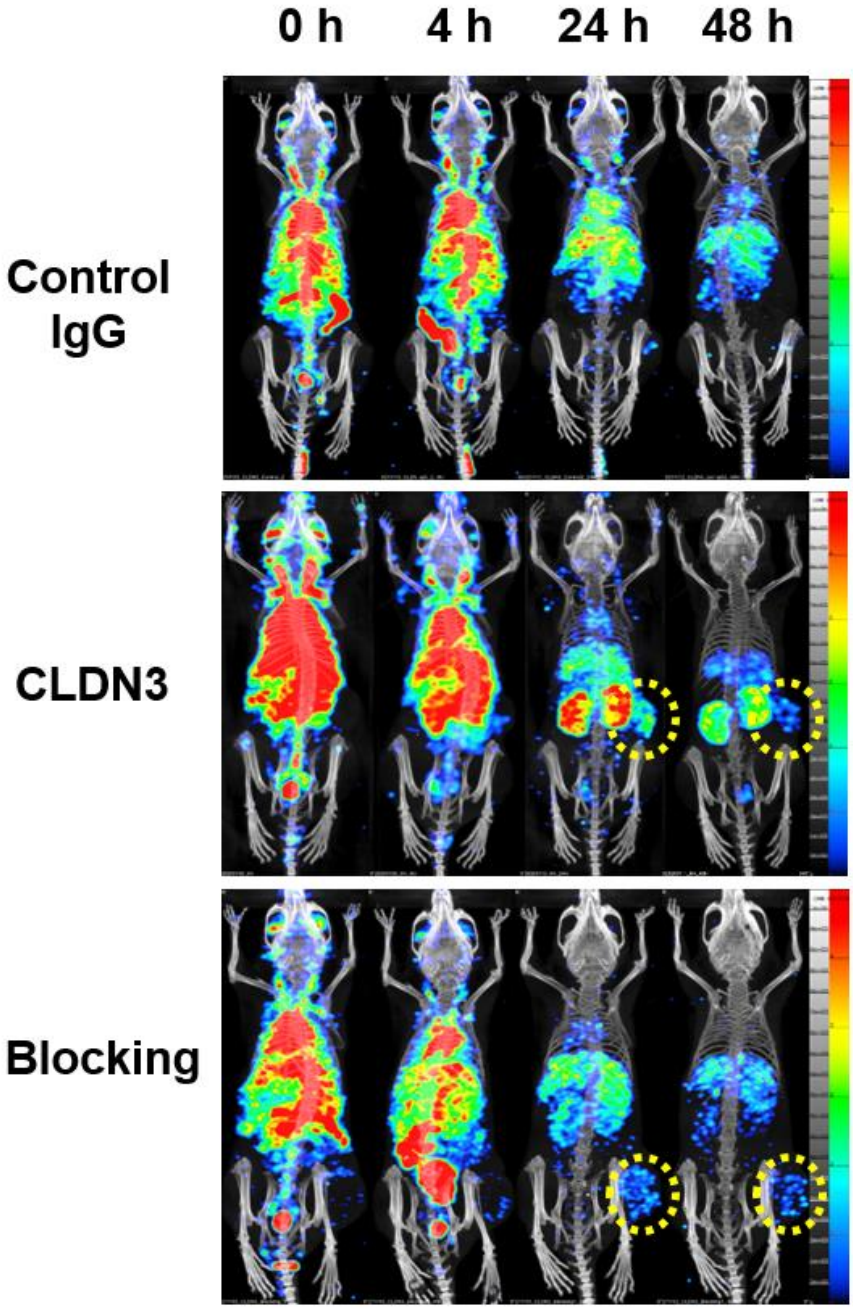


Figure 10. SPECT/CT images of ^{111}In-labeled CLDN3 antibody

Based on the time-dependent imaging, ^{111}In-labeled CLDN3 specific

antibody showed high tumor uptake 24 h post-injection, which is significantly higher than that of other groups. The blocking group injected with unlabeled cold antibody showed lower tumor uptake than that of CLDN3 injected group.

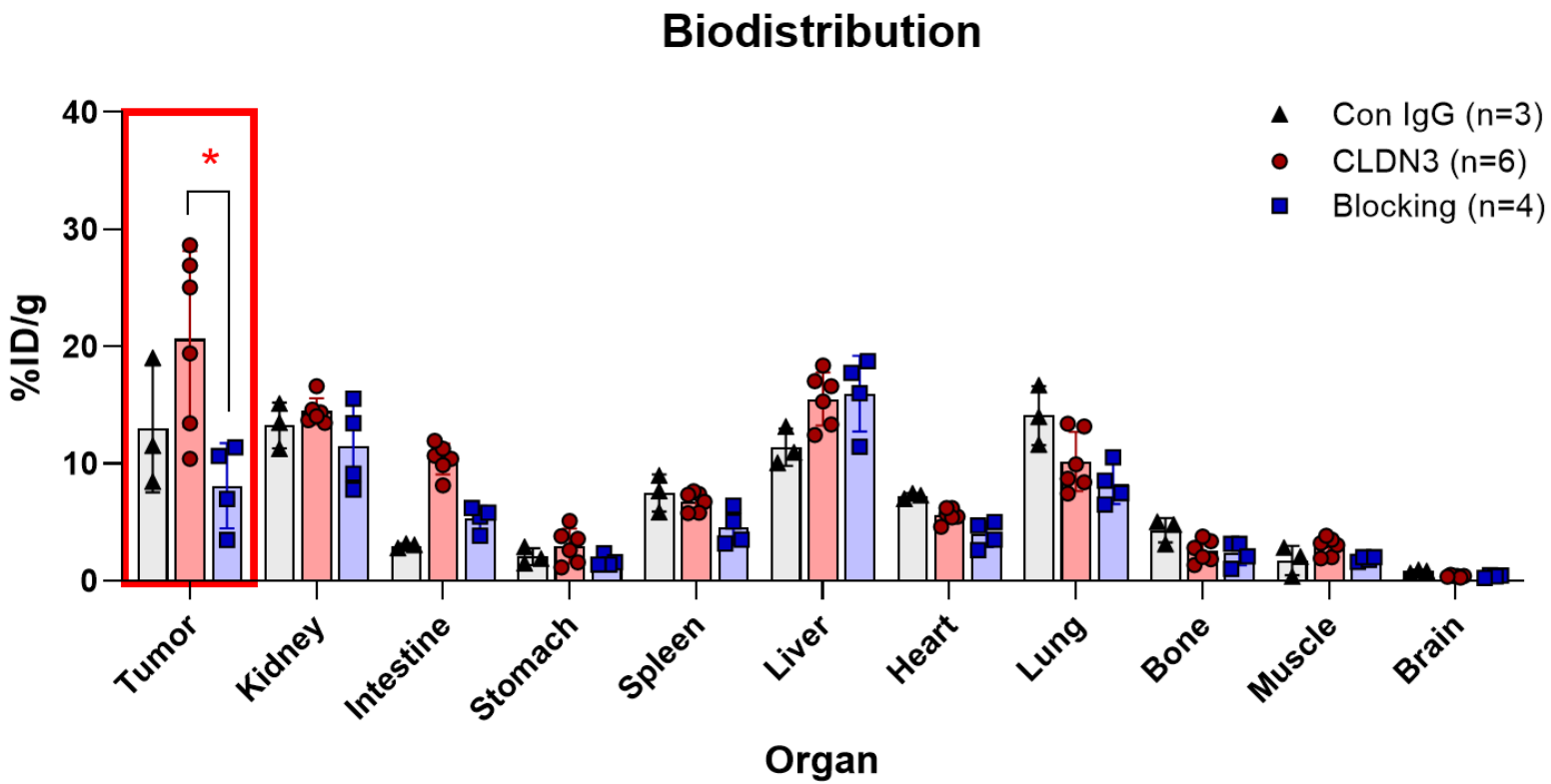

Biodistribution
Con IgG (n=3)
CLDN3 (n=6)
Blocking (n=4)
%ID/g
40
30
20
10
0
*
Tumor
Kidney
Intestine
Stomach
Spleen
Liver
Heart
Lung
Bone
Muscle
Brain
Organ

Figure 11. Biodistribution of [^{111}In]In-NOTA-CLDN3 in mice bearing xenograft OVCAR-3 tumor at 24 h.

Table 2. *In vivo* distribution in OVCAR-3 tumor bearing mice injected with ^{111}In-labeled antibody

%ID/g	Control (n=3)	CLDN3 (n=6)	Blocking (n=4)
Tumor	**13.0 ± 5.4**	**20.6 ± 7.5**	**8.1 ± 3.6**
Kidney	**13.3 ± 1.9**	**14.5 ± 1.1**	**11.5 ± 3.6**
Intestine	**3.0 ± 0.2**	**10.4 ± 1.3**	**5.3 ± 1.0**
Stomach	**2.1 ± 0.7**	**3.0 ± 1.5**	**1.7 ± 0.4**
Spleen	**7.5 ± 1.6**	**6.8 ± 0.8**	**4.5 ± 1.5**
Liver	**11.4 ± 1.6**	**15.5 ± 2.3**	**16 ± 3.2**
Heart	**7.2 ± 0.2**	**5.6 ± 0.6**	**4.0 ± 1.1**
Lung	**14.1 ± 2.5**	**10.2 ± 2.5**	**8.3 ± 1.7**
Bone	**4.3 ± 1.0**	**2.5 ± 0.9**	**2.4 ± 1.0**
Muscle	**1.7 ± 1.3**	**2.9 ± 0.8**	**1.9 ± 0.2**
Brain	**0.8 ± 0.1**	**0.4 ± 0.1**	**0.4 ± 0.1**

Mice injected with ^{111}In-labeled CLDN3-specific antibody showed significantly higher tumor uptake than that of other groups, which was

statistically significant ($p < 0.05$). High uptake was also observed in some normal tissues, such as the kidneys and lungs and liver. Bone, muscle and brain tissue showed lower uptake than other tissues.

DISCUSSION

In many cancers, CLDN3 is localized to cell membranes and shows promise as a cancer therapeutic target. CLDN3 is an attractive target for cancer therapy because it is accessible in cancer cells compared to normal cells. A normal epithelial cell proliferates parallel to the epithelial plane, and access of CLDN3 is restricted. However, in the early stage of epithelial cancer cell formation, the direction of the mitotic spindle is not controlled, and the cell loses the direction of division and proliferates through out-of-plane division, resulting in CLDN3 is exposed outside. As the exposed CLDN3 becomes easily accessible, CLDN3 can be a therapeutic target that can be specifically delivered to tumor tissue for cancer treatment or anticancer drugs. Therefore, in this study, I tried to investigate the possibility as a potential therapeutic diagnostic biomarker targeting CLDN3 by conjugating a therapeutic entity such as pharmacological or radioactive to CLDN3-specific antibody.

Although the role of CLDN3 in tumorigenesis has not yet been elucidated, it is overexpressed in various carcinomas, plays an important

role in tumor progression and metastasis, and is associated with the characteristic TJ dysfunction, the most important feature of epithelial carcinoma [1-6]. Various molecular imaging and antitumor therapies targeting CLDN3 have been developed. Although CPE-based therapeutics that bind to ECL2 of CLDN3 are the best known of those developed to target the CLDN3 protein, there is still a need to effectively target CLDN3 with high specificity and low toxicity. In addition, since CLDN3 overexpression is known to be associated with poor prognosis in many cancer cells, especially ovarian cancer, it has a potential as a useful biomarker for cancer detection and diagnosis in ovarian cancer. Also, it has been reported that other CLDN3 antibodies are being studied as candidates for drug delivery system in ovarian cancer [42]. However, nuclear medicine treatment targeting CLDN3 has not yet been studied. Through this study, effective targeting of the CLDN3 antibody was evaluated, and it is thought to have a potential as a therapeutic target. Furthermore, this is due to the disadvantage that it is difficult to confirm the location or internalization of *in vitro* cells with confocal microscopy images using isotope-conjugated antibodies. Since theses limitation can

be overcome with fluorescence images, this study attempted to perform dual imaging of nuclear medicine and optical imaging simultaneously.

Antibody-drug conjugates are emerging as potential targeted therapies for the delivery of highly cytotoxic drugs to the tumor site. To date, only four known antibody drug conjugates have received FDA approval. Its limitations include a diverse population with different drug-to-antibody ratios and different drug conjugation sites. For this reason, the synthesis of homogenous immunoconjugates is necessary and important. In order to develop an antibody that effectively targets CLDN3 by conjugating a more homogenous and specific therapeutic entity, this study aimed to evaluate the potential of CLDN3-specific antibody as a new antibody therapeutic agent.

In a previous study, it was confirmed that fluorescence-conjugated CLDN3 antibody was intravenously injected into CLDN3 expressing tumor bearing xenograft mice and localized to the tumor site [30-31]. However, other nuclear medicine imaging agents for CLDN3 have not yet been reported. For example, as a representative nuclear medicine gold standard, [^{18}F]FDG PET is generally used for cancer diagnosis, but

it is uptake not only in tumors but also in areas of brain or inflammatory lesion with high glucose uptake, which often leads to false positive results. Antibodies can improve the selectivity and specificity of tumor-to-normal tissues by delivering drugs such as pharmacologic agents, radioisotopes, or photosensitive agents to the tumor. Therefore, it is expected to be a potential as a non-invasive cancer biomarker using antibodies in nuclear medicine.

Antibody conjugation is generally known to be accomplished by a chemical reaction. Recently, many attempts have been made to conjugate specific enzymes through protein engineering. However, the preferred sites for chemical conjugation of antibodies are lysine and cysteine residues, and after reducing the cysteine residues, we used the click reaction, one of the highly efficient experimental techniques, as an antibody conjugation method. The reaction between azide and alkyne shows a high yield, and it can be used more diversely by relatively easily introducing isotopes, fluorescence, or drugs.

In this study, human CLDN3 antibodies could specifically target CLDN3 positive tumors and the CLDN3-overexpressing human ovarian

cancer cell line OVCAR-3 both *in vitro* and *in vivo*. Optical and nuclear images were successfully acquired by conjugating CLDN3 antibodies with a fluorescent dye or radioactive isotope. These observations suggested that the CLDN3 antibody can be used as an effective CLDN3-specific imaging tracer in various molecular imaging modalities. In order to label a CLDN3-specific antibody, it is important to minimize antibody damage. TCEP is widely used as the most effective reductant for cleaving protein disulfide bonds [34]. After cleavage of the disulfide bond of the antibody using TCEP, highly site-specific click chemistry was used to label imaging tracers to label fluorescence and radioisotopes at the same site without damaging the CLDN3 specific Fab portion and preventing non-specific binding.

Although it was successfully detected CLDN3-overexpressing tumors in a mouse model by injecting the CLDN3 antibody labeled with FNR648 fluorescent dye, autofluorescence was probably detected in the intestinal region because the mice were fed a diet containing alfalfa that could be avoided by feeding an alfalfa-free diet. On the other hand, intact antibody clearance has also been reported to be generally up to 168 hours.

However, it is known to show a remarkably high uptake even in 2 to 3 days [40]. Therefore, given the in vivo half-life of antibodies, radioactive isotopes with half-lives of 2–3 days were considered appropriate. Thus, the CLDN3-specific antibody was labeled ^{111}In with a half-life of 2.8 days for SPECT/CT imaging.

In addition, SPECT/CT images of mice showed high kidney uptake because CLDN3 is one of the proteins constituting the tight junction. And it is known to be expressed not only in tumor but also in normal tissues [32-33]. For example, high expression of CLDN3 is found in the ovary, colon, thyroid, and salivary, and there is also some level of CLDN3 expression in kidney, pancreas, and liver [33]. Therefore, there is a possibility of kidney uptake. As shown in the SPECT/CT image in Figure 10, there was little bladder uptake at 24 h, and tumor or kidney uptake seemed high. Also, according to the serum stability test, ^{111}In-labeled antibody was stable up to 96 ho, so it is thought that there was little possibility that a small size such as [^{111}In]In-NOTA.

Also, the safety of CLDN3 targeted therapeutics and selective targeting of tumor tissues should be considered for clinical application.

For example, it has been reported that CLDN18.2-specific chimeric antigen receptor T cells (CAR T cells) have a therapeutic effect on gastric and pancreatic cancer [35-37]. Recently, FDA approved the use of anti-CLDN18.2 CAR T cells in the treatment of CLDN18.2-expressing gastric, pancreatic, and gastroesophageal junction adenocarcinomas. CAR-engineered immune cell therapy will have a good response and will be a good trial for a promising strategy for anticancer therapy [38].

Recently, various therapeutic radioisotopes have been used alone or in combination by conjugation to antibodies. As one of the most suitable nuclides for radioimmunotherapy using antibodies, ^{177}Lu has therapeutic and diagnostic applications as it can furnish non-invasive whole-body images. Further studies are needed to use of CLDN3-specific antibodies as a theranostic tracer. Currently, the efficacy of binding ^{177}Lu to CLDN3-specific antibodies is being tested, and anti-cancer therapies using CLDN3-specific antibodies combined with other therapeutic radioisotopes in CLDN3-positive tumors are planned to be developed and investigated. However, according to the SPECT/CT imaging and biodistribution results, ^{111}In-labeled antibodies had high kidney uptake.

A drug called Gelofusin is known to inhibit normal renal absorption, and it has been reported that it actually reduces renal absorption in vivo [41]. Treatment with drugs such as gelofusin that inhibit renal absorption reduces renal uptake and makes them more effective tumor targets.

REFERENCES

1. Wodarz A, Näthke I. Cell polarity in development and cancer. Nat Cell Biol. 2007;9(9):1016-1024.

2. Shin K, Fogg VC, Margolis B. Tight junctions and cell polarity. Annu Rev Cell Dev Biol. 2006;22:207-235.

3. Martin TA, Jiang WG. Loss of tight junction barrier function and its role in cancer metastasis. Biochim Biophys Acta. 2009;1788(4):872-891.

4. Maeda T, Murata M, Chiba H, Takasawa A, Tanaka S, Kojima T et al. Claudin-4-targeted therapy using Clostridium perfringens enterotoxin for prostate cancer. Prostate. 2012;72(4):351-360.

5. Todd MC, Petty HM, King JM, Piana Marshall BN, Sheller RA, Cuevas ME. Overexpression and delocalization of claudin-3 protein in MCF-7 and MDA-MB-415 breast cancer cell lines. Oncol Lett. 2015;10(1):156-162.

6. Kominsky SL. Claudins: emerging targets for cancer therapy. Expert Rev Mol Med. 2006;8(18):1-11.

7. Günzel D, Yu AS. Claudins and the modulation of tight junction permeability. Physiol Rev. 2013;93(2):525-569.

8. Morin PJ. Claudin proteins in human cancer: promising new targets for diagnosis and therapy. Cancer Res. 2005;65(21):9603-9606.

9. Singh AB, Sharma A, Dhawan P. Claudin family of proteins and cancer: an overview. J Oncol. 2010;2010:541957.

10. Tsukita S, Yamazaki Y, Katsuno T, Tamura A, Tsukita S. Tight junction-based epithelial microenvironment and cell proliferation. Oncogene. 2008;27(55):6930-6938.

11. Ding L, Lu Z, Lu Q, Chen YH. The claudin family of proteins in human malignancy: a clinical perspective. Cancer Manag Res. 2013;5:367-375.

12. Fujita K, Katahira J, Horiguchi Y, Sonoda N, Furuse M, Tsukita S. Clostridium perfringens enterotoxin binds to the second extracellular loop of claudin-3, a tight junction integral membrane protein. FEBS Lett. 2000;476(3):258-261.

13. Kleinberg L, Holth A, Trope CG, Reich R, Davidson B. Claudin upregulation in ovarian carcinoma effusions is associated with poor

survival. Hum Pathol. 2008;39(5):747-757.

14. Konecny GE, Agarwal R, Keeney GA, Winterhoff B, Jones MB, Mariani A et al. Claudin-3 and claudin-4 expression in serous papillary, clear-cell, and endometrioid endometrial cancer. Gynecol Oncol. 2008;109(2):263-269.

15. Lechpammer M, Resnick MB, Sabo E, Yakirevich E, Greaves WO, Sciandra KT et al. The diagnostic and prognostic utility of claudin expression in renal cell neoplasms. Mod Pathol. 2008;21(11):1320-1329.

16. Anwer S, Branchard E, Dan Q, Dan A, Szászi K. Tumor necrosis factor-α induces claudin-3 upregulation in kidney tubular epithelial cells through NF-κB and CREB1. Am J Physiol Cell Physiol. 2021 Apr 1;320(4):C495-C508.

17. Szade J, Kunc M, Pieczyńska-Uziębło B, Świerblewski M, Biernat W, Jassem J et al. Comparison of claudin-3 and claudin-4 expression in bilateral and unilateral breast cancer. Neoplasma. 2021 Mar;68(2):283-289.

18. Ikeda C, Haga H, Makino N, Inuzuka T, Kurimoto A, Ueda T et al. Utility of Claudin-3 in extracellular vesicles from human bile as

biomarkers of cholangiocarcinoma. Sci Rep. 2021 Jan 13;11(1):1195.

19. Mitchell LA, Koval M. Specificity of interaction between clostridium perfringens enterotoxin and claudin-family tight junction proteins. Toxins (Basel). 2010;2(7):1595-1611.

20. Liu JK. The history of monoclonal antibody development - Progress, remaining challenges and future innovations. Ann Med Surg (Lond). 2014;3(4):113-116.

21. Zahavi D, Weiner L. Monoclonal Antibodies in Cancer Therapy. Antibodies (Basel). 2020;9(3):34.

22. Cruz E, Kayser V. Monoclonal antibody therapy of solid tumors: clinical limitations and novel strategies to enhance treatment efficacy. Biologics. 2019;13:33-51.

23. Weiner LM, Surana R, Wang S. Monoclonal antibodies: versatile platforms for cancer immunotherapy. Nat Rev Immunol. 2010;10(5):317-327.

24. Scott AM, Wolchok JD, Old LJ. Antibody therapy of cancer. Nat Rev Cancer. 2012;12(4):278-287.

25. Coats S, Williams M, Kebble B, Dixit R, Tseng L, Yao NS et al.

Antibody-Drug Conjugates: Future Directions in Clinical and Translational Strategies to Improve the Therapeutic Index. Clin Cancer Res. 2019;25(18):5441-5448.

26. Larson SM, Carrasquillo JA, Cheung NK, Press OW. Radioimmunotherapy of human tumours. Nat Rev Cancer. 2015;15(6):347-360.

27. Akbari B, Farajnia S, Khosroshahi SA, Safari F, Yousefi M, Dariushnejad H et al. Immunotoxins in cancer therapy: Review and update. Int Rev Immunol. 2017;36(4):207-219.

28. Chitgupi U, Qin Y, Lovell JF. Targeted Nanomaterials for Phototherapy. Nanotheranostics. 2017;1(1):38-58.

29. Fass L. Imaging and cancer: a review. Mol Oncol. 2008;2(2):115-152.

30. Yang H, Park H, Lee YJ, Choi JY, Kim T, Rajasekaran N et al. Development of Human Monoclonal Antibody for Claudin-3 Overexpressing Carcinoma Targeting. Biomolecules. 2019;10(1):51.

31. Yang H, Le QV, Shim G, Oh YK, Shin YK. Molecular engineering of antibodies for site-specific conjugation to lipid

polydopamine hybrid nanoparticles. Acta Pharm Sin B. 2020;10(11):2212-2226.

32. Choi YL, Kim J, Kwon MJ, Choi JS, Kim TJ, Bae DS et al. Expression profile of tight junction protein claudin 3 and claudin 4 in ovarian serous adenocarcinoma with prognostic correlation. Histol Histopathol. 2007;22(11):1185-1195.

33. Hewitt KJ, Agarwal R, Morin PJ. The claudin gene family: expression in normal and neoplastic tissues. BMC Cancer. 2006;6:186. Published 2006 Jul 12.

34. Kirley TL, Greis KD, Norman AB. Selective disulfide reduction for labeling and enhancement of Fab antibody fragments. Biochem Biophys Res Commun. 2016;480(4):752-757.

35. Jiang H, Shi Z, Wang P, Wang C, Yang L, Du G et al. Claudin18.2-Specific Chimeric Antigen Receptor Engineered T Cells for the Treatment of Gastric Cancer. J Natl Cancer Inst. 2019 Apr 1;111(4):409-418.

36. Ungureanu BS, Lungulescu CV, Pirici D, Turcu-Stiolica A, Gheonea DI, Sacerdotianu VM et al. Clinicopathologic Relevance of

Claudin 18.2 Expression in Gastric Cancer: A Meta-Analysis. Front Oncol. 2021 Mar 4;11:643872.

37. Iwaya M, Hayashi H, Nakajima T, Matsuda K, Kinugawa Y, Tobe Y et al. Colitis-associated colorectal adenocarcinomas frequently express claudin 18 isoform 2: implications for claudin 18.2 monoclonal antibody therapy. Histopathology. 2021 Aug;79(2):227-237.

38. Bębnowska D, Grywalska E, Niedźwiedzka-Rystwej P, Sosnowska-Pasiarska B, Smok-Kalwat J, Pasiarski M et al. CAR-T Cell Therapy-An Overview of Targets in Gastric Cancer. J Clin Med. 2020 Jun 17;9(6):1894.

39. Torres JB, Knight JC, Mosley MJ, Kersemans V, Koustoulidou S, Allen D et al. Imaging of Claudin-4 in Pancreatic Ductal Adenocarcinoma Using a Radiolabelled Anti-Claudin-4 Monoclonal Antibody. Mol Imaging Biol. 2018 Apr;20(2):292-299.

40. Wu AM. Engineered antibodies for molecular imaging of cancer. Methods. 2014 Jan 1;65(1):139-47.

41. Jin ZH, Furukawa T, Sogawa C, Claron M, Aung W, Tsuji AB et al. PET imaging and biodistribution analysis of the effects of

succinylated gelatin combined with L-lysine on renal uptake and retention of ^{64}Cu-cyclam-RAFT-c(-RGDfK-)$_4$ in vivo. Eur J Pharm Biopharm. 2014 Apr;86(3):478-86.

42. Romani C, Cocco E, Bignotti E, Moratto D, Bugatti A, Todeschini P et al. Evaluation of a novel human IgG1 anti-claudin3 antibody that specifically recognizes its aberrantly localized antigen in ovarian cancer cells and that is suitable for selective drug delivery. Oncotarget . 2015 Oct 27;6(33):34617-28.

국문 초록

서론: 밀착 연접 단백질을 이루는 단백질 중의 하나인 클라우딘-3 (CLDN3) 는 상피 또는 내피 세포 시트에서 세포 간 상호작용을 조절한다. 종양 형성 동안 상피 세포가 변형되고 종양 세포가 평면 외 분열을 통해 증식하면서 클라우딘-3 가 외부로 노출된다. 또한 클라우딘-3의 발현은 암 진행과 관련이 있다고 알려져 있으며 대부분의 난소암은 상피성 난소암으로써 클라우딘-3 발현이 높다고 알려져 있다. 이때문에 본 연구에서는 난소암을 표적하는 클라우딘-3 특이적 항체를 새로운 영상 추적자로써 사용할 수 있는 가능성을 확인하고자 하였다.

방법: −SH 그룹을 노출시키기 위해 클라우딘−3 특이적 항체를 환원시킨 후, 클릭 화학기법을 사용하여 방사성 동위원소 [^{111}In] 또는 형광 단백질 FNR648을 접합시켰다. 인간 난소암 OVCAR−3 및 인간교모세포종 U87MG 세포를 각각 클라

우딘-3 양성 및 음성 세포로 사용하였다. 클라우딘-3 단일 클론 항체의 특이성을 확인하기 위하여 유세포 분석을 사용하여 두 세포주에 결합하는 정도를 확인했다. 클라우딘-3 단일 클론 항체를 이용한 생체 내 영상화를 하기 위하여 OVCAR-3 세포를 마우스에 피하 주사하여 이종이식 모델을 확립했다. [^{111}In]을 표지한 클라우딘-3 특이적 항체(370 kBq/50 μL)를 마우스에 정맥내 투여하였다. 투여 24시간 후, 종양을 포함한 장기를 절제하고 γ-카운터로 측정했다. IVIS 광학 이미징 시스템과 SPECT/CT로 이미지를 획득했다.

결과: NOTA-^{111}In과 클라우딘-3 항체-NOTA-^{111}In의 표지 효율은 각각 98.52%와 100% 인 것을 확인하였다. 형광 단백질 FNR648로 표지된 클라우딘-3 항체는 OVCAR-3 및 U87MG의 세포 표면에 각각 83.4% 및 5.7% 특이도로 결합했다. OVCAR-3 종양 이종 이식된 마우스에서 클라우딘-3 IgG1 항체는 주사 후 24시간에 대조군 IgG1 (8.8 ± 2.6 %ID/g)보다 2.5배 더 높은 종양 섭취 (20.4 ±

7.4 %ID/g)를 보였다. 종양에서 클라우딘-3 항체 형광 신호는 주사 후 24시간에 최고조에 달한 것을 보였다.

결론: 본 연구에서는 방사성 동위원소와 형광 단백질을 클라우딘-3 특이적 항체와 성공적으로 접합하였고 특히 마우스 모델에서 OVCAR-3 종양에 대한 항체의 특이적 결합을 확인함으로써 클라우딘-3 특이적 인간 단일 클론 항체가 유용한 치료학적 추적자로 사용될 수 있음을 이중 영상으로 보여주었다.

주요어: 클라우딘-3, 인간 단일 클론 항체, 이중 영상, 인간 난소암

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