Characterization and Evaluation of the Antitumour Activity of a Dual-targeting Monoclonal Antibody against Claudin-3 and Claudin-4

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Abstract. *Background: Because human claudin-3 and claudin-4 (CLDN3 and CLDN4) are overexpressed in a variety of carcinomas, they are promising targets for cancer therapy. The aim of the present study was to generate a dual-targeting monoclonal antibody against CLDN3 and CLDN4 and evaluate its antitumour activity. Materials and Methods: BALB/c mice were immunised with CLDN4-expressing Chinese hamster ovary cells and cell-based screening was performed. The antibody-binding epitope of CLDN3 and CLDN4 and the antitumour activity of the antibody were evaluated. Results: A monoclonal antibody, KM3907 (IgG2a), which recognised CLDN3 and CLDN4, but not CLDN5, CLDN6 and CLDN9, was successfully isolated. The binding assay of KM3907 revealed that KM3907 recognised the extracellular loop 1 of CLDN3 and CLDN4. Mouse human chimeric IgG1 induced antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity in vitro, and treatment with murine KM3907 significantly inhibited tumour formation in SCID mice in vivo. Conclusion: A dual-targeting monoclonal antibody against CLDN3 and CLDN4 is a promising strategy for cancer immunotherapy.*

In recent decades, monoclonal antibodies (mAbs) against cancer have shown enormous potential and efficacy as a new class of drugs. Thus far, nine therapeutic mAbs have been approved in the United States, and over 100 more mAb products are in clinical trials (1). One of the reasons for their success is that mAb drugs possess intrinsic advantages as native immunological molecules: specificity, stability, and Fc-mediated effector functions. In particular, the effector functions of mAbs, including antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), are responsible for their unique mode of action in destroying target cells in comparison to conventional small-molecule drugs against cancer. In addition, recent studies suggest that the effector functions of mAb are important for the therapeutic efficacy of mAbs (2). Human claudin-3 and claudin-4 (CLDN3 and CLDN4) are tetraspanin transmembrane proteins consisting of 220 and 209 amino acids, respectively, and are members of the CLDN family that comprises 24 members (3). The protein structure has been predicted to consist of cytoplasmic *N*- and *C*-termini, four transmembrane domains, and two extracellular loops, EL1 and EL2, from the *N*-terminus, respectively (4). CLDN members form homo- or hetero-binding between adjacent cells and generate a tight junction, which is a key construct for epithelial barrier and defence functions (3). CLDN3, along with CLDN4, also function as receptors for *Clostridium perfringens* enterotoxin (CPE) (5).

Interestingly, several studies have indicated that the expression of CLDN3 and CLDN4 is high in a variety of carcinomas (6, 7), and the high level of expression is related to the poor prognosis for patients with gastric (8), endometrial (9), ovarian (10) and breast (11) cancer. Although the expression of CLDN3 and CLDN4 is detectable in a variety of normal tissues, including kidney and intestinal mucosa, its intensity in cancerous tissues has been shown to be considerably higher than in normal tissues. The role of CLDN3 and CLDN4 in carcinogenesis is not fully understood, but the expression profiles in malignant carcinomas suggest that CLDN3 and CLDN4 are promising targets for cancer therapy. In fact, CPE-related compounds (12, 13) and a monoclonal antibody against CLDN4 (14) have shown promising antitumour efficacy in preclinical models.

The concept of antibody-targeting against two different molecules is a promising approach to enhance the efficacy

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of therapeutic mAbs. In fact, combinations of monoclonal antibodies, such as trastuzumab (anti-Her2) and bevacizumab (antivascular endothelial growth factor) (15), and artificial bispecific antibodies, which are generated by protein engineering directed against two different antigens (16), have shown superior therapeutic potential in preclinical or clinical settings. The aim of the present study was to generate a dual-targeting mAb against CLDN3 and CLDN4 and to evaluate its therapeutic potential against tumours. To isolate an objective mAb, human CLDN3-, CLDN4-, CLDN5-, CLDN6-, and CLDN9-expressing Chinese hamster ovary (CHO) cells (CLDN/CHO) (14) were generated and used as immunogen or target cells in cell-based screening. CLDN5, CLDN6, and CLDN9 share high homology with CLDN3 and CLDN4, and it is therefore necessary to deselect antibodies that are cross-reactive to these CLDN molecules.

Materials and Methods

Mice and cell lines. BALB/c mice and C.B.-17/Icr-scid Jcl (SCID) were obtained from CLEA Japan, Inc. (Tokyo, Japan) and maintained under pathogen-free conditions. All experiments were performed in conformity with institutional guidelines and in compliance with national laws and policies.

CHO cell line, DG44, was kindly provided by Dr. Lawrence Chasin (Columbia University). Breast cancer cell line, MCF-7 (HTB-22), and ovarian cancer cell line, MCAS (JCRB 0240), were obtained from the American Type Culture Collection (Manassas, VA, USA) and the Japanese Collection of Research Bioresources (Osaka, Japan), respectively.

Establishment of CLDN/CHO cells. CLDN/CHO cells were generated as described previously (14). In brief, myc/his tag sequence was connected to the 3' end of CLDN open reading frame by polymerase chain reaction (PCR) and then the myc/his-tagged cDNA was cloned into pKANTEX93 vector (17). The expression vector was introduced DG44 cells *via* electroporation. G418-resistant clones were selected and a high-producing cell was isolated by single-cell cloning. The appropriate expression of transfection gene was confirmed by reverse transcriptase-PCR and anti-tag antibodies (14).

To generate mouse/human chimeric CLDN3-expressing CHO cells, the chimeric cDNA was generated by site-directed mutagenesis into human *CLDN3* cDNA using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and was then subjected to the above procedure.

Isolation of dual targeting mAb against CLDN3 and CLDN4. Six-week-old female BALB/c mice (CLEA) were immunised four times with 1×10^7 CLDN4/CHO with *Bordetella pertussis* adjuvant. The spleens were removed 3 days after the final injection of the antigen, and 2×10^8 splenocytes were fused with 2×10^7 mouse myeloma cell line, P3X63Ag8U.1 (CRL-1597, obtained from the American Type Culture Collection), in the presence of polyethylene glycol 1000 (Junsei, Tokyo, Japan) to prepare antibody producing hybridoma. Cultured hybridoma cells in wells showing anti-CLDN3 and anti-CLDN4 antibody activity were screened by an 8200 Cellular Detection System (Applied Biosystems, Tokyo, Japan), using CLDN4/CHO and CLDN3/CHO as target cells. After cloning twice with limited dilution, a stable clone was obtained. The immunoglobulin class and subclass were determined with anti-mouse isotype-specific antibodies (Zymed, San Francisco, CA, USA).

Production and purification of mouse human chimeric KM3907. The heavy- and light-chain variable region cDNAs from hybridoma cells producing KM3907 were isolated by PCR and cloned into pKANTEX93 vector for production of mouse human chimeric IgG1 antibody. The vector was then introduced into DG44 cells *via* electroporation and the transfected cells were grown in serum-free EX-CELL302 (JRH Bioscience, Lenexa, KS, USA). Chimeric KM3907 antibody was then purified from the supernatant using Prosep-A column (Nihon Millipore, Tokyo, Japan).

Flow cytometry (FCM). For the analysis of KM3907 binding to cell surface molecules, cells were detached with 0.02% EDTA solution (Nacalai Tesque, Kyoto, Japan) and then stained with 2.9 μg/ml of KM3907 or control mouse IgG2a (Dako, Tokyo, Japan). The reactivity was detected by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig antibody (Dako, Tokyo, Japan). Stained cells were then analysed using an EPICS XL-MCL FCM (Beckman Coulter, Tokyo, Japan).

ADCC. Peripheral blood mononuclear cells (PBMCs) were separated from the peripheral blood of healthy donors using Lymphoprep (Axis-Shield, Oslo, Norway) and used as effector cells. Aliquots of the target cells (1×10^4 cells/well) and effector cells (2.5×10^5 cells/well, effector/target ratio of 25/1) were put into 96-well plates and incubated with different concentration of chimeric KM3907 for 4 h at 37˚C. After centrifugation, the released lactate dehydrogenase (LDH) in the supernatant was detected by Cytotox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA). Percentage-specific lysis was calculated from sample counts according to the formula:

$$\% \text{ Cytotoxicity}=100\times(E-S)/(M-S)$$

where, E is the experimental release (count in the supernatant from target cells incubated with antibody and effector cells), S is the spontaneous release (count in the supernatant from target cells incubated with medium alone) and M is the maximum release (count released from target cells lysed with Triton ×-100).

Blood donors were randomly selected from healthy volunteers registered at Kyowa Hakko Kirin Co., Ltd. All donors gave written informed consent prior to participation.

CDC. Target cells (5×10^4) were incubated with different concentrations of chimeric KM3907 and human serum (Sigma-Aldrich, Tokyo, Japan) as the source of complement at a dilution of 1:6 in supplemented RPMI-1640 medium for 2 h at 37˚C in 96-well flat-bottomed plates. After incubation, the cell proliferation reagent WST-1 (Roche Diagnostics, Tokyo, Japan) was added (15 μl/well) and the plates were further incubated for 4 h to detect the live cells. The absorbance (wavelength: 450-650 nm) of the formazan dye produced by metabolically active cells of each well was detected on an Emax plate reader (Molecular Devices, Union City, CA, USA). Cytotoxicity was calculated using the following formula:

$$\% \text{ Cytotoxicity}=100\times[1-(E-S)/(M-S)]$$

where, *E* is the experimental absorbance (cells incubated with antibody and complement), *S* is the spontaneous absorbance (medium and complement alone) and *M* is the maximum absorbance (cells incubated with medium and complement alone).

In vivo antitumour activity. CLDN3/CHO (1×10^7), CLDN4/CHO (1×10^7) and MCAS (1×10^6) cells were injected subcutaneously into 7-week-old male SCID mice (CLEA). With regard to the inoculation of CLDN3/CHO and CLDN4/CHO, recipient mice were pretreated with anti-asialo GM1 antibody (Wako, Osaka, Japan) three days before the inoculation. After cell injection, 10 mg/kg of murine KM3907 or phosphate-buffered saline (PBS) were injected intraperitoneally twice a week for three weeks (total: six injections). Tumour volume was calculated using the following equation:

Tumour volume (mm^3)=0.5×(major diameter)×(minor diameter)^2

Statistical analysis. Results are expressed as means or mean±standard deviation (SD). Statistical calculations were performed using Microsoft Excel 2003 (Microsoft, Redmond, WA, USA). The statistical significance of differential findings between the experimental groups was determined by two-tailed unpaired Student's *t*-test.

Results

Isolation of a dual-targeting monoclonal antibody against CLDN3 and CLDN4. To isolate the anti-CLDN3 and anti-CLDN4 mAb, first BALB/c mice were immunised with CLDN4/CHO to generate hybridoma cells producing the anti-CLDN4 mAb and then the cross-reactive mAb against CLDN3 was selected by cell-based screening (Applied Biosystems 8200). Moreover, CLDN5, CLDN6 and CLDN9/CHO cells were used as negative targets to exclude cross-reactive antibodies against these CLDN molecules. Several candidates were screened and deselected by specific reactivity against CLDN4 (did not react with CLND3) or cross-reactivity against CLDN5, CLDN6 and CLDN9. Finally, an objective mAb, KM3907 (IgG2a), which bound to CLDN3 and CLDN4/CHO but did not bind to CLDN5, CLDN6, CLDN9, or vector/CHO, was successfully isolated (Figure 1). The binding activity of KM3907 was also confirmed against a CLDN3- and CLDN4-expressing breast cancer cell line, MCF-7 (12) (Figure 1). Interestingly, KM3907 exhibited superior binding potency against CLDN4/CHO rather than CLDN3/CHO in FCM analysis (data not shown). Therefore, KM3907 may possess higher affinity for CLDN4 than for CLDN3.

In addition, immunoprecipitation assays showed that KM3907 bound to CLDN3 and CLDN4 proteins (data not shown); however, the binding was not detected in Western blotting using KM3907 as a primary antibody. These results suggest that KM3907 may recognise the conformational structure of CLDN3 and CLDN4.

KM3907 recognises the EL1 domain of CLDN3 and CLDN4. Next, the study aimed to identify the extracellular domain of CLDN3 and CLDN4 recognised by KM3907. Since KM3907 bound to human but not to mouse CLDN3, mouse/human CLDN3 chimera were generated and the binding of KM3907 was evaluated against these chimeras. Three types of CLDN3 chimera, mCL3-EL1/2 (mouse EL1 and mouse EL2), mCL3-EL1 (mouse EL1 and human EL2), and mCL3-EL2 (human EL1 and mouse EL2), were constructed by introducing site-directed mutations into human CLDN3, and expression CHO cells were then generated. On incubation of chimeric CLDN3/CHO with KM3907, KM3907 was found to bind to mCL3-EL2 but not to mCL3-EL1/2 or mCL3-EL1 (Figure 2). However, the same strategy was not used to identify the CLDN4 extracellular domain recognised by KM3907 because KM3907 showed cross-reactivity against human and mouse CLDN4. Therefore, artificial CLDN4 was generated, in which the domains were exchanged between CLDN4 and CLDN6 (Figure 2) and the binding of KM3907 was evaluated. In FCM analysis, KM3907 showed binding to CLDN4/6 (CLDN4 EL1 + CLDN6 EL2) but not to CLDN6/4 (CLDN6 EL1 + CLDN4 EL2). These results suggest that KM3907 recognised the EL1 domains of CLDN3 and CLDN4.

Sequence comparison among the predicted extracellular domains of CLDN molecules showed that the homology

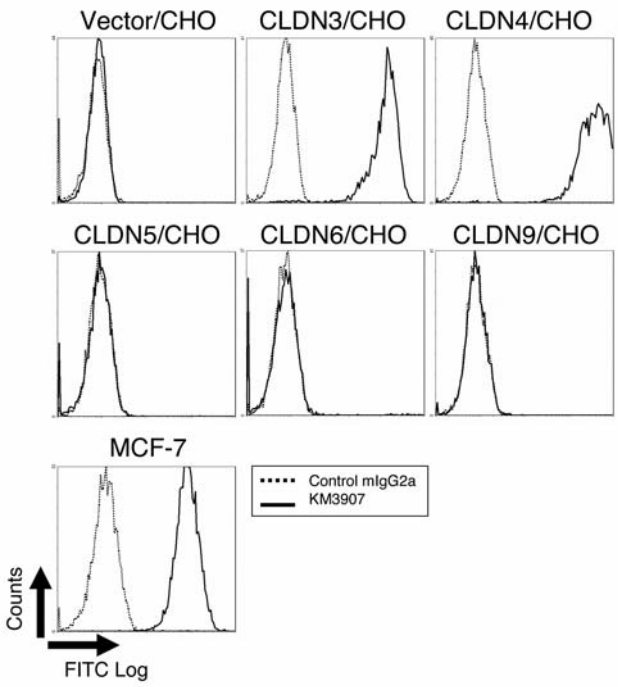


Figure 1. *KM3907 binds to CLDN3 and CLDN4, but not to CLDN5, CLDN6 and CLDN9. CLDN-expressing CHO cells (CLDN3/CHO, CLDN4/CHO, CLDN5/CHO, CLDN6/CHO and CLDN9/CHO) and vector-transfected CHO (Vector/CHO) were generated. These target cells and MCF-7, a human breast cancer cell line, were incubated with control mouse IgG2a (dotted line) or KM3907 (solid line) and the binding was detected by a FITC-conjugated anti-mouse antibody.*

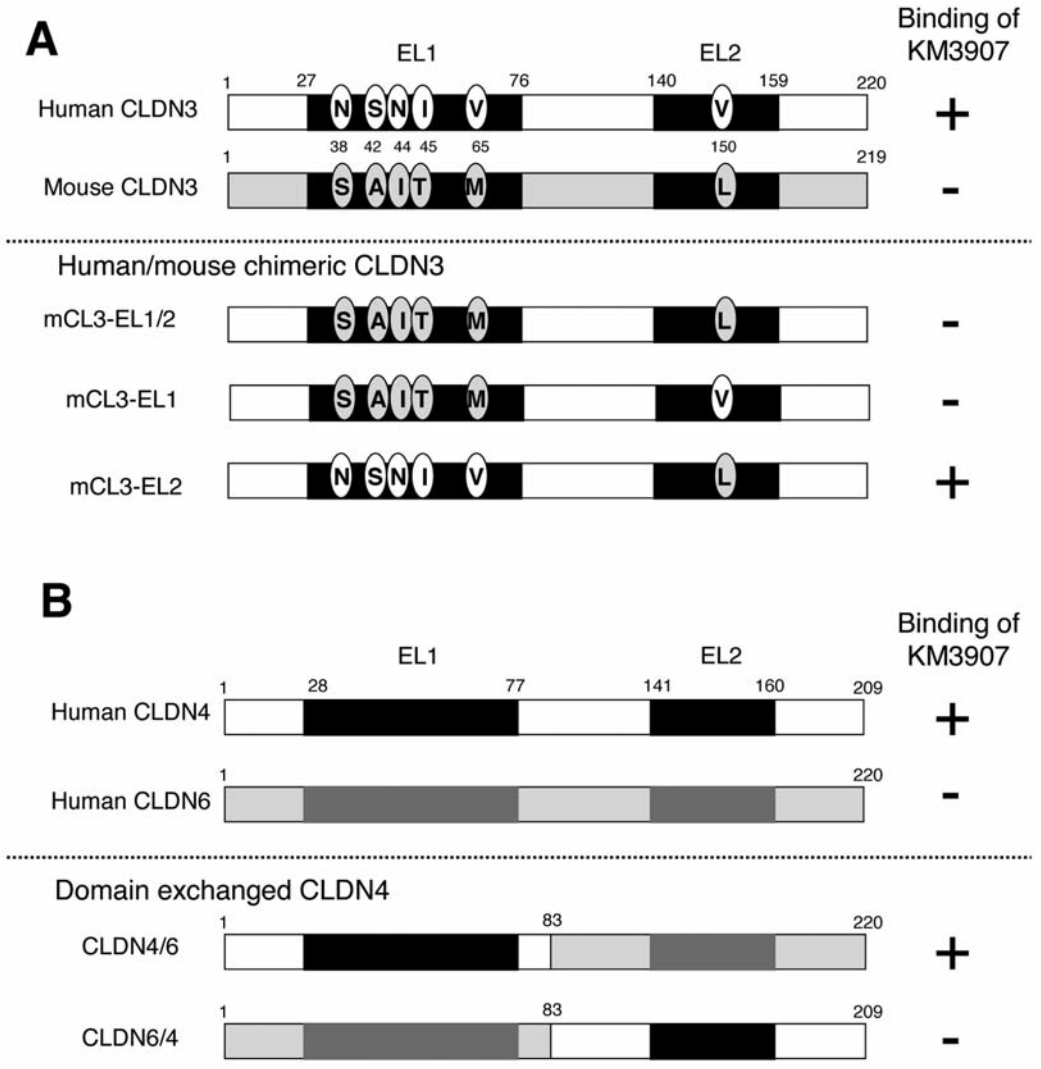
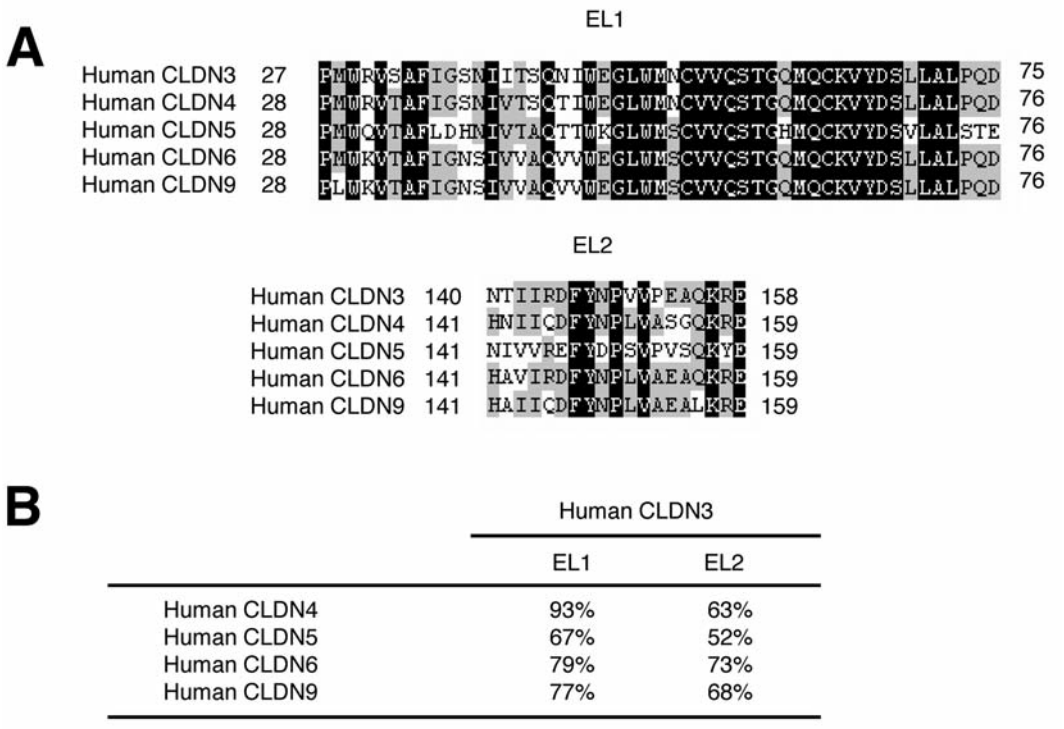


Figure 2. *KM3907 recognises the EL1 domain of CLDN3 and CDLN4. Mouse/human chimeric CLDN3 (mCL3-EL1/2, mCL3-EL1 and mCL3-EL2) were constructed by introducing N38S/S42A/N44I/I45T/V65M into human EL1 and/or V150L into human EL2 with site-directed mutagenesis. The expressing CHO cells were established and incubated with KM3907. Binding was evaluated by using an FITC-conjugated anti-mouse antibody (A). CLDN molecules with domains exchanged between CLDN4 and CLDN6 at amino acid position 83 from the N-terminus (CLDN4/6 and CLDN6/4) were constructed, and the expressing CHO cells were generated. KM3907 reacted with the CHO cells, and its binding was evaluated by using an FITC-conjugated antibody (B).*

between the EL1 domains of CLDN3 and CLDN4 exceeded 90% (Figure 3). In contrast, the sequence homology between the extracellular domains of CLDN3 and those of other CLDN molecules was not as high (50-80%) (Figure 3). These findings suggest that the high sequence homology of the EL1 domains of CLDN3 and CLDN4 and their poor homology with the domains of other CLDN molecules are the reasons for the successful isolation of a dual-targeting mAb against CLDN3 and CLDN4.

Evaluation of the antitumour activity of KM3907. By using a PCR method, the variable regions of heavy and light chains from KM3907 hybridoma cells were cloned and the expression vector of mouse human chimeric IgG1 with a human IgG1 Fc domain (cKM3907) was constructed. CHO/DG44 was used for the production of cKM3907, which was purified by using a Protein-A column. The eluent contained intact IgG (150 kDa) at a purity of more than 90% as confirmed by SDS-PAGE (data not shown). The specificity and affinity for CLDN3- and CLDN4-expressing cells were nearly equivalent to those for murine KM3907 (data not shown).

Human PBMC-mediated ADCC of cKM3907 were evaluated against CLDN3/CHO, CLDN4/CHO, MCF-7 and



B

	Human CLDN3	
	EL1	EL2
Human CLDN4	93%	63%
Human CLDN5	67%	52%
Human CLDN6	79%	73%
Human CLDN9	77%	68%

Figure 3. *A: Alignment analysis of the predicted EL1 and EL2 domains of CLDN. Predicted EL1 and EL2 domain sequences of human CLDN3, CLDN4, CLDN5, CLDN6, and CLDN9. Perfectly matched and highly conserved amino acid sequences are highlighted in black and gray, respectively. The numbers on both sides indicate the amino acid position from the N-terminus. B: Homology (%) between the predicted extracellular domains of human CLDN3 and the other CLDN molecules.*

vector/CHO. At an effector/target ratio of 25:1, cytotoxicity was measured by activity of LDH released from destroyed targets. ADCC activity of cKM3907 was detected in CLDN3/CHO, CLDN4/CHO and MCF-7, but not in vector/CHO (Figure 4). In addition, cKM3907 induced dose-dependent CDC activity against CLDN3/CHO, CLDN4/CHO and MCF-7 by using human complement but not against vector/CHO (Figure 5). In this evaluation, ADCC and CDC activities of cKM3907 were higher against CLDN4/CHO than against CLDN3/CHO. The difference in the expression level of the target molecule in both cells was not large, as determined by using an anti-tag antibody (data not shown). Therefore, the result may be attributed to the differences in the affinity of KM3907 for CLDN3 and CLDN4.

Next, the *in vivo* antitumour efficacy of murine KM3907 was evaluated against CLDN3- and CLDN4-expressing cells. To this end, CLDN3/CHO, CLDN4/CHO and MCAS, which is a human ovarian cancer cell line and expresses CLDN3 and CLDN4 proteins (data not shown), were inoculated subcutaneously into SCID mice (day 0), and then 10 mg/kg of KM3907 or PBS were injected intraperitoneally twice a week. Prior to the experiment, it was confirmed that all tumour cell-inoculated mice had actually developed tumour under the experimental condition by preliminary testing (data not shown). Consequently, the tumour formation in these mice was evaluated and the tumour volumes were calculated at days 32-35. While tumour formation and growth were confirmed in all PBS-treated groups, KM3907-treated mice did not show visible and measurable tumour formation derived from CLDN3/CHO, CLDN4/CHO and MCAS (Table I).

Discussion

The present study used cell-based screening to successfully isolate a dual-targeting mAb that recognised CLDN3 and CLDN4 but did not bind to CLDN5, CLDN6 and CLDN9. To the Authors' knowledge, this is the first time that a mAb possessing unique dual specificity against human CLDN3 and CLDN4 has been generated. In addition, a mouse human chimeric mAb, cKM3907, was generated and its antitumour activity was evaluated *in vitro*. The mAb cKM3907 induced ADCC and CDC activities against CLDN3- and CLDN4-expressing target cells. Furthermore, murine KM3907 showed superior prophylactic efficacy against tumour formation in SCID mice. These results suggest that mAb therapy targeting CLDN3 and CLDN4 is a promising therapeutic approach for cancer.

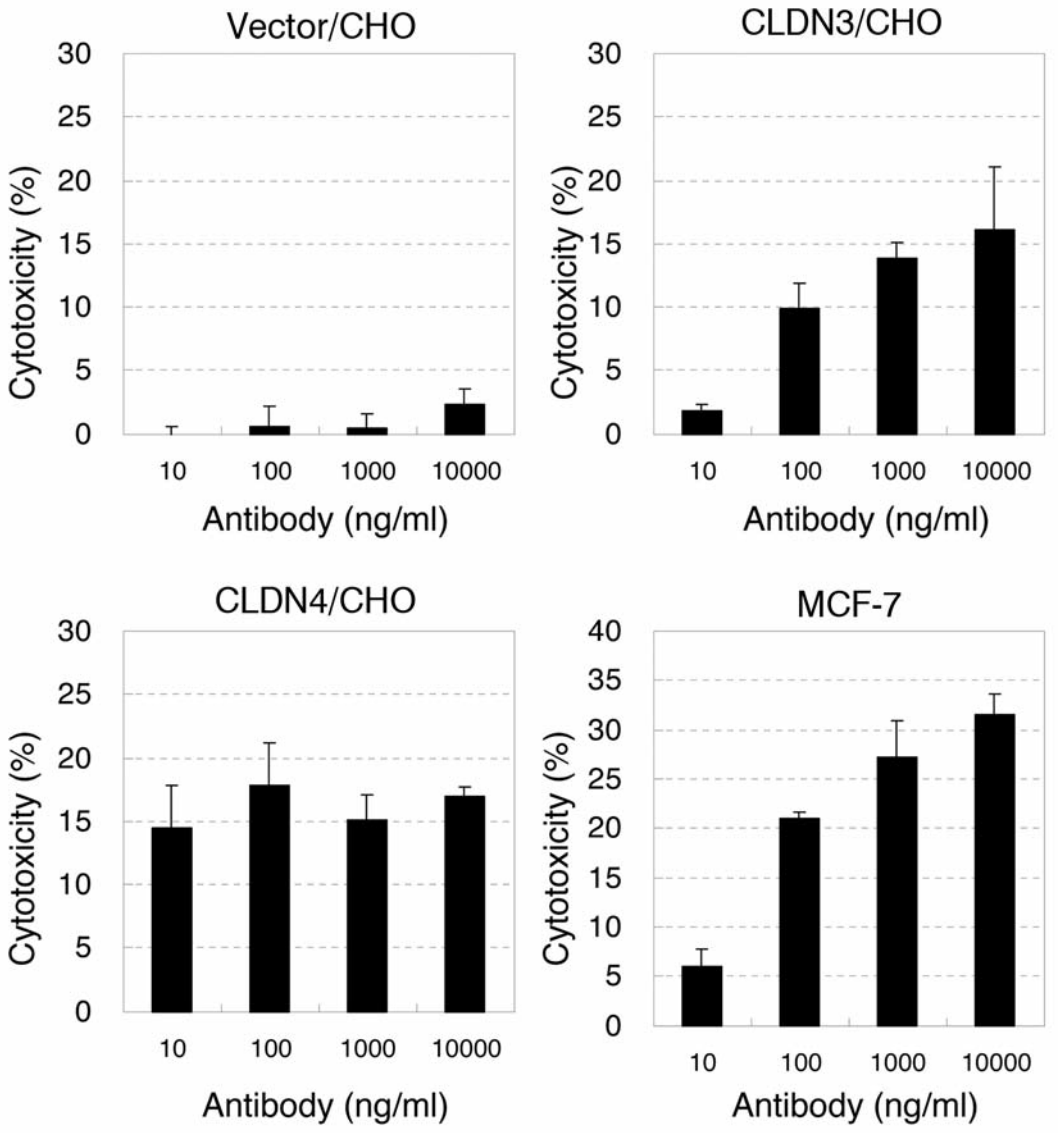


Figure 4. *ADCC activity of cKM3907. Human peripheral blood mononuclear cells were purified from healthy donors and used as effector cells. Target cells (vector/CHO, CLDN3/CHO, CLDN4/CHO and MCF-7) were incubated with effector cells (effector/target=25/1) and cKM3907. The released lactate dehydrogenase activity was measured and cytotoxicity (%) was determined (N=3).*

Dual-targeting mAb, which recognises different target molecules, is principally expected to demonstrate superior efficacy in comparison with a mAb specific for a single target (16). In fact, KM3907 showed promising antitumour activity not only against CLDN3- but also CLDN4-expressing cells both *in vitro* and *in vivo*. Moreover, in a preliminary comparison of the effector functions of cKM3907 and an anti-CLDN4-specific mAb, which recognises the EL2 domain of CLDN4 (14), cKM3907 tended to induce higher ADCC and CDC against CLDN3- and CLDN4-expressing targets than did chimeric anti-CLDN4-specific mAb (data not shown). Although these are preliminary results, the dual-targeting mAb against CLDN3 and CLDN4 may be expected to exert superior efficacy as compared to a specific monoclonal antibody against CLDN3 or CLDN4 alone. Such a dual-targeting mAb should find applications in a clinical setting because CLDN3 and CLDN4 are frequently detected in the same carcinoma specimens (6, 7).

On the basis of the binding of KM3907 to mouse/human chimeric CLDN3 and artificial domain-exchanged CLDN4, it is concluded that KM3907 recognised the EL1 domains of CLDN3 and CLDN4. Since the homology between the EL1 domains of CLDN3 and CLDN4 was extremely high (>90%), while that with the domains of CLDN5, CLDN6 and CLDN9 was relatively low, the EL1 domain may serve as an acceptable

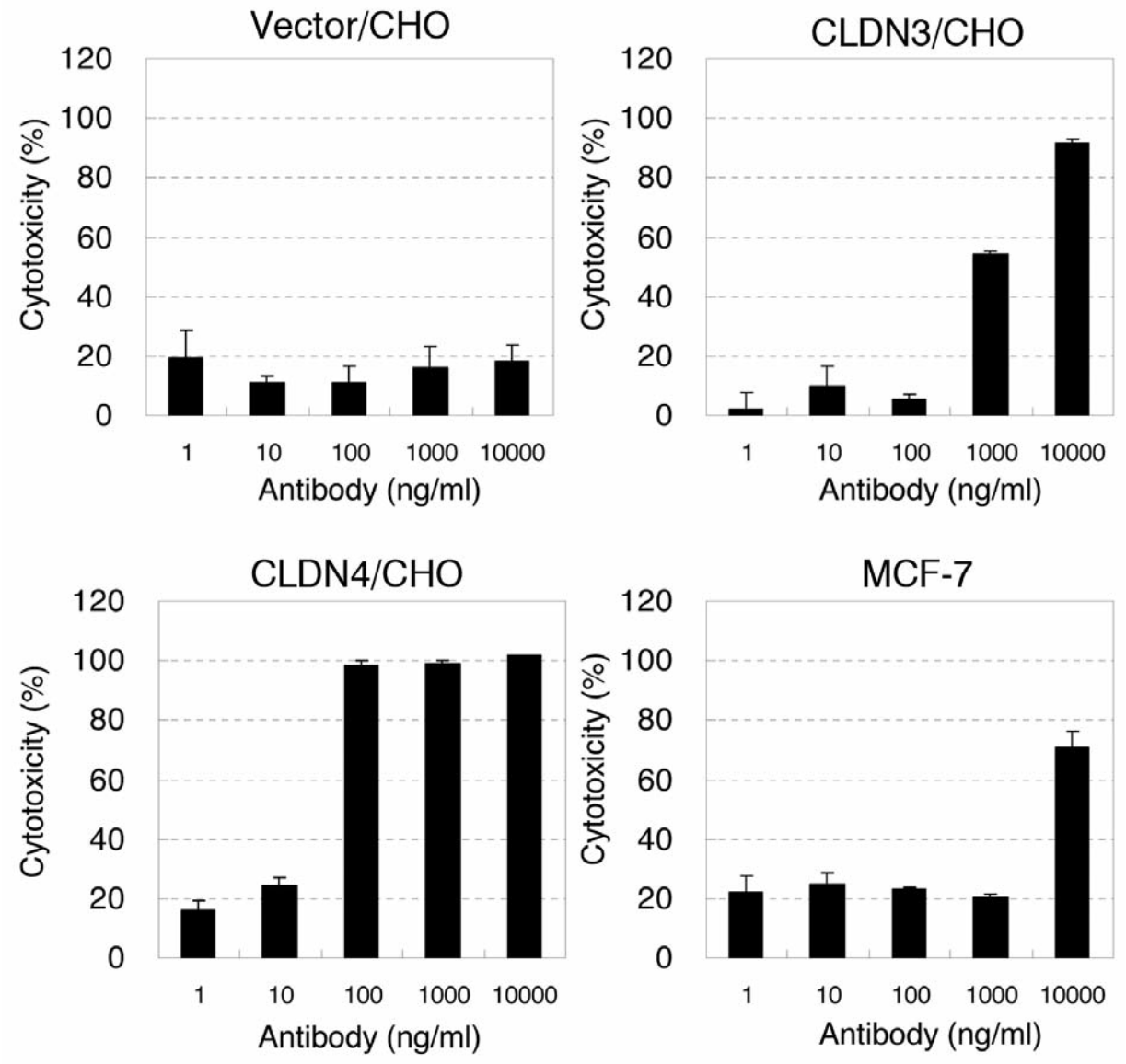


Figure 5. *CDC activity of cKM3907. Target cells (vector/CHO, CLDN3/CHO, CLDN4/CHO, and MCF-7) were incubated with human complement (16.7%) and cKM3907. Cell viability was measured by a WST-1 assay and cytotoxicity (%) was determined (N=3).*

Table I. *In vivo antitumour activity of KM3907. CLDN3/CHO (1×10^7), CLDN4/CHO (1×10^7), and MCAS (1×10^6) were inoculated subcutaneously into SCID mice (day 0). After inoculation, 10 mg/kg murine KM3907 or PBS was administered intraperitoneally twice a week from day 0 (N=5), and the tumour size (mm^3) was measured at days 32-35. There were statistically significant differences (*$p<0.01$) in the tumour sizes as compared to PBS-treated group (control).*

Target cell	Treatment	Number of tumour-bearing mice	Tumour volume (mm^3) (days 32-35)
CLDN4/CHO	PBS	5/5	1398.0±542.0*
	KM3907	0/5 (Day 35)	-
CLDN3/CHO	PBS	5/5	1106.1±415.0*
	KM3907	0/5 (Day 35)	-
MCAS	PBS	5/5	747.1±388.9*
	KM3907	0/5 (Day 32)	-

epitope for a dual-targeting mAb against CLDN3 and CLDN4. Interestingly, CPE, which is a protein toxin produced by *Clostridium perfringens* and is known to bind to CLDN3 and CLDN4, recognises the EL2 domain of CLDN3 (18). It was also reported that CPE bound not only CLDN3 and CLDN4 but also CLDN6, CLDN7, CLDN8 and CLDN14 (18). Although further evaluation of specificity is required, the isolated KM3907 may be a superior agent for targeting CLDN3 and CLDN4 and thus may be a valuable tool for therapeutic or diagnostic applications in cancer therapy.

In an oncological setting, CPE has been well validated and has shown promising antitumour efficacy both *in vitro* and *in vivo* (12). CPE is a single polypeptide of 319 amino acids and causes cytolysis of CLDN3- and CLDN4-expressing cells. In addition, engineered CPE-related agents, including toxin-conjugated CPE fragments, have also shown superior antitumour effects against several epithelial carcinoma types (13). However, because CPE may be treated as a foreign substance and may induce severe immunological reactions in the human body, its usage may be restricted to local administration. The use of mAbs may overcome the limitations of the use of CPE-related agents and mAbs may serve as a new tool for targeting CLDN3 and CLDN4. Because humanisation technology can reduce the immunogenicity of mAb, humanised mAb is expected to show superior pharmacological properties in a clinical setting.

CLDN3 and CLDN4 molecules are well characterized and highly expressed in a variety of epithelial carcinomas, including pancreatic, ovarian, cervical, breast, gastric and colorectal cancer (6, 7). Although further evaluation is required, the antitumour potential of KM3907 indicates that a dual-targeting mAb against CLDN3 and CLDN4 can exert therapeutic effects against these refractory carcinomas. At the same time, since both molecules are expressed in a variety of epithelial tissues, including kidney and intestinal mucosa (19, 20), the effects of this dual-targeting mAb against these healthy tissues may need careful evaluation.

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