Targeting claudin-4 enhances chemosensitivity of triple negative breast cancer

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Abstract

Background Triple negative breast cancer (TNBC) possesses highly aggressive phenotype, treatment with limited options, and a poor prognosis. In this study, we examined the therapeutic effect of anti-claudin (CLDN)-4 extracellular domain antibody, 4D3, on TNBC. **Methods** The expression of CLDN4 and CLDN1 in invasive ductal carcinoma (IDC) was examined in 78 IDCs (from 2004 to 2009 in a single center). CLDN expression and the effect of 4D3 on proliferation were examined in human IDC cell lines MCF-7 (luminal subtype) and MDA-468 (TNBC). **Results** In IDC cases, CLDN1 had lower expression than CLDN4 and correlated with histological grade. In contrast, expression of CLDN4 correlated with histological grade, receptor subtype, and stage. CLDN4 expression in the two cells was at the same level. In both cells, paclitaxel (PTX)-induced growth suppression was enhanced by 4D3. Furthermore, 4D3 increased both intracellular PTX concentration (in both cells) and apoptosis. In the mouse model, 4D3 promoted the antitumor effect of PTX on subcutaneous tumors and reduced lung metastasis. The combination of PTX and 4D3 reduced M2 macrophages and mesenchymal stem cells in the tumor. 4D3 also reduced stemness of the tumors in association with increase in the intratumoral pH. Moreover, concurrent treatment of 4D3, PTX, or tamoxifen; or with PTX and tamoxifen in MDA-468 also showed the same level of antitumor activity and survival as MCF-7. Furthermore, in bone metastasis model, combination of PTX and bisphosphonate with 4D3 promoted tumor growth in both cells. **Conclusions** CLDN4 targeting of the antibody facilitated existing therapeutic effects.

Background

Breast cancer is the third leading cause of cancer death in Japanese women [1]. The common histological type, invasive ductal carcinomas (IDC) frequently express hormone receptors (luminal subtype) and/or human epidermal growth factor receptor-2 (HER2, Her2 subtype)[2, 3]. In contrast, approximately 15% of IDCs express none of estrogen receptor (ER), progesteron receptor (PgR), or HER2, which is designated as triple-negative breast cancer (TNBC) [4]. TNBCs possess more malignant phenotypes than the usual IDCs, with rapid growth and a high frequency of recurrence and metastasis [5]. The treatment of breast cancer commonly includes a combination of surgery, radiation, chemotherapy, hormone therapy, and targeted therapy against HER2 [6]. However, TNBCs that is lacking in molecular therapy targets are also currently lacking in any effective molecular therapy; which therefore result in an associated high mortality and poor prognosis [4, 7]. Identifying novel molecular targets is an important issue for TNBCs. Several molecular therapy candidates of TNBCs have been reported. TNBCs frequently express epidermal growth factor receptor (EGFR) at high levels and mutant p53 [8, 9]. EGFR targeting, using cetuximab, improves the progression-free survival but not the overall survival in TNBC [10]. We have reported that a suppressive receptor of the renin-angiotensin system, MAS1, is expressed frequently in TNBCs. Angiotensin 1-7 activating MAS1 might be a relevant molecular target for TNBCs [11].

Tight junction is one of the intercellular adhesion structures that control the paracellular traffic [12]. In the epithelium, tight junction acts as a barrier or fence between the luminal space and the epithelium [12, 13], which prevents the permeation of harmful substances and the leakage of physiologically active substances. Thus, tight junctions play a role in maintaining the microenvironment by inhibiting the invasion of anticancer drugs into tumor tissues, and promoting intratumoral retention of growth factors in cancer [14-16].

CLDN4 is a major component of the epithelial tight junction [17]. CLDNs forms a family of 27 isoforms with very homologous structures [12, 13, 18]. CLDN4 exhibits high expression in epithelial tissues, and the expression is also seen in epithelial malignant tumors [19-22]. We have studied the availability of CLDN4 targeting in cancer therapy by preparing the antibody specific to the extracellular domain of CLDN4 [16]. The anti-CLDN4 extracellular domain antibody (4D3) decreases the barrier function in cancer, promotes permeation of anticancer agents, and enhances its antitumor effect [14-16].

Paclitaxel (PTX) is a first-line therapeutic drug in the treatment of breast cancer [23]. PTX exerts its antitumor activity by promoting the polymerization and stabilization of tubulin to form microtubules, stacking cell cycle at the G2/M phase, which causes apoptosis [24]. PTX is also applied as treatment for TNBCs with immune check point inhibitors [25, 26].

In this study, we investigated the effect of 4D3 on IDC, especially TNBC, and assessed the efficacy in combination treatment with PTX and PD-1 inhibitor.

Methods

Surgical specimens We performed the pathological diagnosis and reviewed clinical data of 78 patients diagnosed with IDCs in the Department of Molecular Pathology, Nara Medical University from 2004 to 2009. We selected luminal subtype (47 cases), Her2 subtype (23 cases) and triple negative subtype (TNBC, 8 cases). Because no written informed consents were obtained, any identifying informational data were removed from the samples before analysis to protect privacy strictly (unlinkable anonymization). All procedures were done in accordance with the Ethical Guidelines for Human Genome/Gene Research which is issued by the Japanese Government. The procedures were approved by the Ethics Committee of Nara Medical University (approval number 937). Cell lines MCF-7 and MDA-468 human IDC cell lines were purchased from Dainihon Pharmaceutical Co. (Tokyo, Japan). Cells were maintained at 37°C in 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) which was supplemented with 10% fetal bovine serum (FBS). Cell growth was assessed using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) dye assay (Wako Pure Chemical Corp. Ltd., Osaka, Japan), as previously described [27]. A pH 7.0 DMEM was prepared from regular DMEM (pH 7.4) adjusted by the addition of HCl. **Antibody and reagents** The anti-human CLDN4 antibody 4D3 was developed by

immunizing rats with a plasmid vector encoding human CLDN4, which recognized the extracellular domains of CLDN4 [16]. The anti-human CLDN1 extracellular domain antibody 2C1 was also developed by the same method [28]. PTX (Wako) and estradiol (LKT Labs Inc., St. Paul, MN, USA) were purchased. Apoptosis of cells was determined by staining with the Hoechst 33258 fluorescent dye (Wako). The number of apoptotic cells was counted by examining 1,000 stained cells [27]. Animals BALB/c nude mice (4 weeks old, male) were purchased from SLC Japan (Shizuoka, Japan). All mice were housed 2 or 3 per wide cage. The holding room was maintained at 23°C, 50% humidity, with 12-hours light/12-hours dark cycle. The mice were euthanized at the end of experiments by cervical dislocation under anesthesia with isoflurane (3%). All experiments were performed according to the institutional guidelines and were approved by the Committee for Animal Experimentation of Nara Medical University (approval number 11725), which was accordant with the current regulations and standards of the Ministry of Health, Labor, and Welfare, Japan. To establish a model for subcutaneous tumors, cells (1×10^7) were inoculated subcutaneously into the subscapular areas of nude mice. Then, with five mice in each group, PTX (10 mg/kg body weight [BW]) and/or 4D3 (1 mg/kg BW, diluted with saline) were injected concurrently into the peritoneal cavity on days 1, 3, and 7. Tumor size was observed each week. According to the institutional humane endpoint for animal experiments, moribund mice were euthanized. For anti-estrogen therapy, tamoxifen citrate (TAM, 500 mg per mouse in peanut oil, daily, s.c., LKT Labs) was administrated [29]. Lung metastasis model IDC cell suspension (1×10^6 cells/50 μ l of PBS) was injected into the caudal vein. PTX (10 mg/kg BW) and 4D3 (1 mg/kg BW) were also administrated (i.p.) on days 1, 3, and 7. Bone metastasis model Under inhalation anesthesia with 3% isoflurane (WAKO), percutaneous intraosseal injection was performed by drilling a 26-gauge needle into the tibia proximal to the tuberositas tibia, then IDC cell suspension (5×10^5 cells/20 μ l of phosphate buffered saline [PBS]) was inserted [30]. Mice were treated with bisphosphonate (BP, zoledronic acid, WAKO, 100 μ g/kg in 100 μ l PBS) [31], PTX (10 mg/kg BW) and 4D3 (1 mg/kg BW) administrated (i.p.) on days 1, 3, and 7. In vivo imaging of tumor IDC cell were labeled with VivoTrack 680 (ParkinElmer Inc., Waltham, MA, USA). A mouse was examined by Clairvivo OPT in vivo imager (Shimazu, Kyoto, Japan) under anesthesia [16]. Fluorescence intensity was calculated with software equipped in the imager. Intratumoral pH Tumors were penetrated by 18G needle, through which lumen a fine needle probe of pH meter (Chemical Instruments, Co. LTD., Hachioji, Japan) was inserted into tumors under anesthesia. pH was monitored for 5 min to calculate the mean value in one site. The measurement was performed at five sites for each tumor. Representative pH was a mean of values in the five sites. Immunohistochemistry Thin sections (4- μ m) of paraffin-embedded specimens were immunohistochemically stained with 0.2 μ g/mL of 4D3 or 2C1 by a previously described immunoperoxidase technique [32]. Secondary antibodies conjugated with peroxidase (Medical and Biological Laboratories, Nagoya, Japan) were used (0.2 μ g/mL). Color development were performed with diamine benzidine hydrochloride (DAKO, Glostrup, Denmark). A counterstain was done with Meyer's hematoxylin (Sigma). Immunopositive cells at the cytoplasmic membrane were counted. Staining strength was scored from 0 to 3 (a score of 1 was used to describe the expression level in normal pancreatic duct epithelium). The staining index was calculated as the staining strength score multiplied by the staining area (%) [14-16]. For a negative control, non-immunized rat IgG (Santa-Cruz Biotechnology, Santa-Cruz, CA, USA) was used as the primary antibody. Immunoblot analysis Whole-cell lysates were

prepared as previously described [33]. Lysates (20 µg) were used to immunoblot analysis using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 12.5%), and then transferred electronically onto nitrocellulose filters. The filters were incubated with primary antibodies and peroxidase-conjugated secondary antibodies (Medical and Biological Laboratories). Anti-tubulin antibody was used as a loading standard (Oncogene Research Products, Cambridge, MA, USA). The signals were visualized with an Enhanced Chemiluminescence Western-blot detection system (Amersham, Aylesbury, UK). Antibodies for CLDN4 (4D3), CLDN1 (2C1), and ER (Proteintech Group Inc., Rosemont, IL, USA) were used as primary antibodies. Reverse transcription-polymerase chain reaction (RT-PCR) To assess human CLDN4 mRNA expression, RT-PCR was performed with total RNA (0.5 µg) extracted using an RNeasy kit (Qiagen, Germantown, MD, USA). The primer sets were as follows; mouse Batf2 (M1 macrophage), forward, 5'-AGC ACG AAT CCT TGG AGA AA AA-3' and reverse, 5'-GTT CCT GGC AGC CAT TGT AT-3' (National Center for Biotechnology Information [NCBI] Reference Sequence: BC024521.1); mouse Fizz1 (M2 macrophage), forward, 5'-CCC TTC TCA TCT GCA TCT CC-3' and reverse, 5'-CAG TAG CAG TCA TCC CAG CA-3' (NCBI Reference Sequence: AF316397.2); mouse CD73 (mesenchymal stem cell), forward, 5'-CCT CTC AAA TCC AGG GAC AA-3' and reverse, 5'-TTT GGA AGG TGG ATT TCC TG-3' (NCBI Reference Sequence: L12059.1); which were synthesized by Sigma Genosys, Ishikari, Japan. PCR products were electrophoresed with a 2% agarose gel and stained with ethidium bromide. The β-actin (ACTB) mRNA was also amplified as an internal control (GenBank Accession No. NM_001101). Enzyme-linked immunosorbent assay (ELISA) ELISA kits were used to measure the concentrations of PTX (anti-5-PTX antibody-derived ELISA, Absolute Antibody, UK), according to the manufacturers' instructions. Statistical analysis Statistical significance was calculated using chi-square test and Kruskal-Wallis test with InStat software (GraphPad, Los Angeles, CA, USA). Survival analysis was performed using the Kaplan-Meier method along with the log-rank test (SPSS Statistics, IBM Japan, Tokyo, Japan). Statistical significance was defined as a two-sided p-value of < 0.05.

Results

Expressions of CLDN4 and CLDN1 in human IDCs

Expressions of CLDN4 and CLDN1 were examined by immunohistochemistry in IDCs (Fig. 1). In a solid-tubular/luminal type IDC case, CLDN4 was located at the cytoplasmic membrane in cancer cells (Fig. 1A). The CLDN4 expression level was higher than that of CLDN1. In a scirrhous/triple negative IDC case, CLDN4 expression was retained at the cytoplasmic membrane (Fig. 1B). In contrast, CLDN1 expression was not detected.

We next compared the CLDN4 expression with clinicopathological parameters in the 78 cases (Table 1). CLDN4 expression index was associated with histological differentiation, receptor subtype, nodal metastasis (pN), and pathological stage. In contrast, CLDN1 expression index was associated with histological differentiation, and nodal metastasis (pN), but not with receptor subtype. Notably CLDN4 expression in TNBC was higher than those in luminal or HER2 subtypes.

Effect of 4D3 in human IDC cell lines

Human IDC cell lines, MDA-468 (triple negative subtype) and MCF-7 (luminal subtype), were examined with the expressions of CLDN4, CLDN1, and ER with or without E2 treatment (Fig. 2A). MDA-468 cells expressed CLDN4 at higher levels than MCF-7 cells. In contrast, CLDN1 expression was detected at lower levels in the two cell lines than that of CLDN4. ER expression was detected in only MCF-7. CLDN4 and CLDN1 expression were not altered by E2 treatment in MDA-468 cells. In contrast, both expressions were decreased in E2-treated MCF-7 cells.

We previously established 4D3 antibody for targeting CLDN4 in cancer cells [16]. MDA-468 and MCF-7 cells were treated with 4D3 and compared with those treated with 2C1 [28] with and without PTX treatment (Fig. 2B and C). In both cell lines, 2C1 showed no growth inhibition alone, and no enhanced PTX effect. In contrast, 4D3 enhanced PTX-induced growth inhibition at each PTX concentration in both cell lines.

Consistent with enhanced drug permeation into tumor tissues owing to impaired tight junction [16], intracellular PTX levels were found to increase in 4D3-treatment in the two cell lines (Fig. 2D). PTX-induced apoptosis was increased by 4D3 in the treatment involving both cell lines.

Effect of 4D3 on antitumoral effect of PTX

We examined the antitumor effect of 4D3 on the two cell lines treated with PTX in nude mice (Fig. 3). In a subcutaneous tumor model, the antitumor effect of PTX when treated in combination with 4D3 was 0.54 times and 0.65 times promoted in MCF-7 and MDA-468 cells, respectively (Fig. 3A). Similarly, in a lung metastasis model, the lung weight decreased by 0.63 times and 0.58 times in MCF-7 and MDA-468 cells, respectively (Fig. 3B).

In our previous report, 4D3 abrogated tumor microenvironment to reduce intratumoral accumulation of growth factors [16]. In the MDA-468 tumors, we examined the effects of 4D3 on tumor stromal cell population and cancer cell stemness (Fig. 3C, D). PTX treatment increased mRNA expression of mouse M2 macrophage and MSC, and decreased the expression of mouse M1 macrophage. In contrast, 4D3 alone decreased mRNA expression of mouse M2 macrophage and MSC, and increased the expression of mouse M1 macrophage. Moreover, 4D3 abrogated the alteration of the stromal cell population induced by PTX by concurrent treatment. In tumor cells, mRNA expression of stem cell markers, CD133 and CD44 was increased by PTX treatment. In contrast, 4D3 alone decreased expressions of CD133 and CD44 and abrogated PTX-induced expressions of CD133 and CD44 by concurrent treatment.

We next examined the tumor microenvironment by measuring pH of the tumor tissue. By 4D3 treatment, tumor pH was elevated from weak acidic condition to neutral condition (Fig 3E). To elucidate the effect of circumstantial pH on stemness, expressions of CD133 and CD44 was examined in cells cultured in media of pH 7.0 and pH 7.4 (Fig. 3F). Expressions of CD133 and CD44 were higher in pH 7.0 than that in pH 7.4 in both cell lines.

Effect of 4D3 on combination treatment of PTX with TAM

We examined the effect of 4D3 on combination treatment of PTX with TAM using mouse subcutaneous tumor model (Fig. 4A-D). In MCF-7, TAM or PTX alone showed an antitumor effect; however, the combination of both enhanced the effect (Fig. 4A). Furthermore, the combined use of 4D3 enhanced the effects of TAM or PTX and further enhanced the combined effect of TAM and PTX. On the other hand, in MDA-468, TAM alone was not effective, and the combination of TAM and PTX was not different from PTX alone (Fig. 4B). However, the combined use of 4D3 promoted the antitumor effect of PTX, and the same effect as seen in the case of TAM and PTX combination in MCF-7 was obtained.

When examining mouse survival in a similar mouse model with inoculation of MCF-7, the 50% survival periods were 26, 33, and 43 days in mice treated with none (control), PTX and TAM combination, and the three-way combination of PTX, TAM, and 4D3, respectively. Also in the case of MDA-468, 50% survival periods were not significantly prolonged at 35 and 45 days in mice treated with none (control) or PTX and TAM combination, respectively. On the other hand, 50% survival period was significantly extended to 53 days in the three-way combination. A significant prolongation of survivals were found in the three-way combination in both cell lines.

Effect of 4D3 on combination treatment of PTX with BP

Finally, the combined effect of BP (zoledronic acid) with PTX and antibody was examined using a mouse bone metastasis model (Fig. 4E, F). In both MCF-7 and MDA-468 cell lines, there was no anti-tumor effect with BP alone, but an additional effect with PTX was observed. Furthermore, the combination of 4D3 enhanced the antitumor effect in both PTX and the combination of PTX and BP.

Discussion

In the present study, we examined the therapeutic effect of targeting CLDN4 to IDC, particularly TNBC. When the relationship between CLDN4 expression in IDCs and clinicopathological parameters was examined, a correlation was found between stage and nodal metastasis. Furthermore, when correlations of CLDN4 expression with clinicopathological factors were compared between TNBCs and non-TNBCs (luminal and HER2 subtypes), CLDN4 expression in TNBC was higher than for non-TNBC and also correlated strongly with cancer progression. From this, CLDN4 was considered to be more suitable as a molecular target for TNBCs.

Interestingly, CLDN4 expression in non-TNBCs inversely correlated with stage and nodal metastasis, which is similar to that reported in undifferentiated type gastric cancer

[14]. Contrary to the differentiated type gastric cancer, undifferentiated type gastric cancer shows increased, non-assembled (not integrated into tight junction) CLDN4, which is associated with stemness via an integrin signal [14]. Since CLDN4 is known as an epithelial marker [12, 13, 18], downregulation of CLDN4 in non-TNBCs might be associated with epithelial-mesenchymal transition (EMT) [14].

In contrast to non-TNBCs, CLDN4 expression was correlated with primary tumor expansion and nodal metastasis, which is similar to that in bladder cancer [16], colorectal cancer [15], and differentiated type gastric cancer [14]. In such tumors, CLDN4 overexpression might provide an isolated tumor microenvironment mediated through the tight junction, which retains epithelial growth factor (EGF) and vascular endothelial growth factor (VEGF) in the tumor, and inhibits the permeation of anti-cancer drugs [14-16]. In TNBCs, we observed a novel barrier function that the tight junction retained lactic acid in the tumor, which lowers intratumoral pH. Tissue pH was elevated by 4D3 treatment from acidic to neutral in the environment. Cancer cells release lactic acid into the cancer microenvironment by the Warburg effect and changes it into acidic [34]. Acidity in the environment affects the activity of immune cells [35, 36]. We analyzed the alterations in immune cell property in cancerous tissues by 4D3 treatment, which was examined by the expression of immune cell marker genes. As a result, M2 macrophages and MSCs decreased and M1 macrophages increased by 4D3 treatment. M2 macrophages and MSCs are known to promote the metastatic potential and resistance of cancer cells against anticancer drugs [37-39]. When cancer cells were co-cultured with MSCs and pH was changed from an acidic environment to neutral environment and stemness decreased in our data, treatment with 4D3 alone showed no significant antitumor effect in *in vitro* but modest in mouse models. 4D3 also possesses antibody-dependent cellular cytotoxicity activity [16]. In the *in vivo* environment, a stronger antitumor effect than *in vitro* might be induced including the immunostimulatory effect by the destruction of the cancer microenvironment.

Comparing the expression of the epithelial CLDNs between CLDN4 and CLDN1, the expression level of CLDN4 was higher and correlation with clinicopathological factors was also observed. In contrast, the expression level was low in CLDN1 and less correlation with clinicopathological factors was found. From this, CLDN4 was considered to be a better molecular target than CLDN1. It was previously reported that CLDN4 is upregulated in bladder cancer, colon cancer, gastric cancer, and pancreatic ductal cancer, and correlates with cancer progression [14-16, 40]. CLDN4 is considered to be a molecular target with broad efficacy in epithelial malignancies.

We previously reported that 4D3 promoted the antitumor effect of CDDP, 5-fluorouracil (5-FU), and FOLFIRINOX in bladder cancer, colon cancer, gastric cancer and pancreatic ductal cancer [14-16]. Here, we examined the effects of 4D3 in breast cancer cells, and the use of 4D3 promoted the antitumor effect of PTX in both TNBC and luminal subtype. In the luminal subtype, the anti-tumor effect of TMX was promoted by 4D3 and a synergistic effect

with PTX was observed. In contrast, in TNBC, a combination with 4D3 and PTX showed an effect comparable to the PTX and TMX combination in the luminal subtype. Furthermore, when the effect of the 4D3 on the effect of BP was examined in the bone metastasis model, the growth in bone metastasis was suppressed by the 4D3 combination in both TNBC and luminal subtype. Thus, 4D3 was found to promote its effect on any of the anticancer agent, anti-hormone agent, and BP.

Here, we examined the efficacy of 4D3 using a nude mouse model. However, since this 4D3 does not recognize mouse CLDN4, its toxicity to the host could not be evaluated. It is necessary to evaluate the toxicity of 4D3 using an effective model in the future.

From the above findings, 4D3 is considered to be an effective molecular targeting therapeutic agent for IDC, particularly for TNBC. Application in future clinical trials is desired.

Conclusion

CLDN4 targeting using the specific antibody against the extracellular domain facilitated existing therapeutic effects to IDC, especially TNBC.

Abbreviations

TNBC, triple negative breast cancer; CLDN, claudin; IDC, invasive ductal carcinoma; PTX, paclitaxel; HER2, human epidermal growth factor receptor 2; ER, estrogen receptor; PgR, progesteron receptor; EGFR, epidermal growth factor receptor; TAM, tamoxifen; BP, bisphosphonate; MSC, mesenchymal stem cell; FU, fluorouracil

Declarations

Ethics approval and consent to participate: All experimental procedures were performed in accordance with the Ethical Guidelines for Human Genome/Gene Research issued by the Japanese Government and were approved by the Ethics Committee of Nara Medical University (approval number 937).

Consent for publication: As written informed consent was not obtained, any identifying information was removed from the samples prior to analysis, in order to ensure strict privacy protection (unlinkable anonymization).

Availability of data and material: All data generated or analyzed during this study are included in this published article.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: YL analyzed the patient data and histological examination and in vivo examinations. SK performed in vitro and in vivo examinations. TS, KG, and TM assisted in vivo examinations. HO and SM assisted in vitro examinations. RFT and YN assisted histological examination. MK provided CLDN antibodies. HK designed and supervised the study. All authors read and approved the final manuscript.

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Tables

Table 1. Expression of CLDN4 and CLDN1 in 78 invasive ductal carcinomas

Parameters ³⁾	n Expression index ¹⁾			
	CLDN1	<i>P</i> ²⁾	CLDN4	<i>P</i> ²⁾
Total	7843 ± 5		209 ± 8	
Age				
-50	3545 ± 7	NS	214 ± 10	NS
51-	4344 ± 6		209 ± 12	
Histology				
Papillo-tubular	11 63 ± 23	0.0488	148 ± 25	0.0393
Solid tubular	35 29 ± 7		218 ± 11	
Scirrhous	32 49 ± 6		206 ± 15	
Subtype				
Luminal	47 45 ± 6	NS	204 ± 12	0.0041
HER2	23 40 ± 8		197 ± 15	
Triple negative	8 42 ± 14		296 ± 11	
Histological grade				
G1	16 25 ± 8	NS	241 ± 17	NS
G2	48 43 ± 7		207 ± 11	
G3	14 63 ± 14		178 ± 16	
Pathological stage				
1	13 46 ± 10	NS	215 ± 18	0.0127
2a	52 49 ± 7		222 ± 10	
2b	9 19 ± 9		137 ± 24	
3	4 10 ± 4		190 ± 46	
Primary tumor				
pT1	15 41 ± 9	NS	209 ± 16	NS
pT2	57 45 ± 7		210 ± 10	
pT3-4	6 28 ± 11		200 ± 26	
Nodal metastasis				
pN0	68 48 ± 6	0.0098	218 ± 8	0.0021
pN1-2	10 9 ± 3		143 ± 27	

1) The staining index was calculated as the staining strength score (0 to 3) multiplied by the staining area (%).

2) P value was calculated by Kuruskal-Wallis test.

3) Clinicopathological parameters were classified according to AJCC [41]. pT1, tumor ≤ 2 cm in greatest dimension; pT2, tumor ≤ 5 cm in greatest dimension; pT3, tumor > 5 cm; pT4, Tumor of any size with direct extension to the chest wall and/or to the skin (ulceration or skin nodule; pN0, no regional lymph node metastasis; pN1, metastases in 1–3 axillary lymph nodes; and/or in internal mammary nodes with metastases detected by sentinel lymph node biopsy but not clinically detected; pN2, metastases in 4–9 axillary lymph nodes; or in clinically detected internal mammary lymph nodes in the absence of axillary lymph node metastases; stage 1, pT1/pN0; stage 2a, pT1/pN1 or pT2/pN0; stage 2b, pT2/pN1 or pT3/pN0; stage 3, pT1-2/pN2, pT3/pN1-2, pT4/any pN or any pT/pN3.

Table 2. Expression of CLDN4 in TNBC, luminal and HER2 subtypes

	TNBC		Luminal		HER2	
Parameters ⁴⁾	n	CLDN4 ¹⁾ p ²⁾	n	CLDN4 ¹⁾ p ²⁾	n	CLDN4 ¹⁾ p ²⁾ p ³⁾
Histology						
Papillo-tubular	0-	0.00387	181 $\pm$ 24	NS	4 213 $\pm$ 20	NS NS
Solid tubular	5300 $\pm$ 1		21199 $\pm$ 19		9 171 $\pm$ 29	
Scirrhou	3223 $\pm$ 23		19212 $\pm$ 15		10229 $\pm$ 22	
Histological grade						
G1	0-	-	11232 $\pm$ 22	NS	5 262 $\pm$ 19	NS NS
G2	7267 $\pm$ 17		27201 $\pm$ 14		14191 $\pm$ 22	
G3	1300		9 166 $\pm$ 20		4 175 $\pm$ 19	
Pathological stage						
1	1300	NS	8 196 $\pm$ 17	0.00874	4 230 $\pm$ 44	0.0489NS
2a	3300 $\pm$ 1		32220 $\pm$ 12		16207 $\pm$ 17	
2b	3223 $\pm$ 23		6 125 $\pm$ 32		1 80	
3	1300		1 195		2 185 $\pm$ 85	
Primary tumor						
pT1	1300	0.0357	10191 $\pm$ 10	NS	4 230 $\pm$ 44	NS 0.0233
pT2	4300 $\pm$ 1		35209 $\pm$ 81		18194 $\pm$ 17	
pT3-4	3223 $\pm$ 23		2 130 $\pm$ 14		1 270	
Nodal metastasis						
pN0	7267 $\pm$ 18	0.00984	0213 $\pm$ 68	0.0092	21214 $\pm$ 15	0.0221NS
pN1-2	1300		7 136 $\pm$ 75		2 90 $\pm$ 10	

1) The staining index was calculated as the staining strength score (0 to 3) multiplied by the staining area (%).

- 2) P value was calculated by Kuruskal-Wallis test on expression indexes.
- 3) P value was calculated by χ^2 test on case numbers.
- 4) Clinicopathological parameters were classified according to AJCC [41]. pT1, tumor $\leq$ 2 cm in greatest dimension; pT2, tumor $\leq$ 5 cm in greatest dimension; pT3, tumor $>$ 5 cm; pT4, Tumor of any size with direct extension to the chest wall and/or to the skin (ulceration or skin nodule; pN0, no regional lymph node metastasis; pN1, metastases in 1-3 axillary lymph nodes; and/or in internal mammary nodes with metastases detected by sentinel lymph node biopsy but not clinically detected; pN2, metastases in 4-9 axillary lymph nodes; or in clinically detected internal mammary lymph nodes in the absence of axillary lymph node metastases; stage 1, pT1/pN0; stage 2a, pT1/pN1 or pT2/pN0; stage 2b, pT2/pN1 or pT3/pN0; stage 3, pT1-2/pN2, pT3/pN1-2, pT4/any pN or any pT/pN3.

Figures

Figure 1

Figure 1

Expression of CLDN4 and CLDN1 in invasive ductal carcinomas An immunohistochemical evaluation to identify CLDN4 (A, C) and CLDN1 (B, D) at the cytoplasmic membrane of cancer cells using anti-CLDN4 antibody, 4D3, and anti-CLDN1 antibody, 2C1, respectively. (A, B) Solid-tubular type, nuclear grade (NG) 2, pT1N0, stage 1, luminal subtype. (C, D) Scirrhous type, NG3, pT2N1, stage 2, and triple negative subtype. Bar, 100 μ m.

Figure 2

Figure 2

Effects induced by the 4D3 antibody on paclitaxel (PTX)-induced antitumoral effect in human invasive ductal ductal carcinoma cell lines (A) mRNA expressions of CLDN4, CLDN1, and estrogen receptor (ER) with or without estradiol (1 nM) in MDA-468 and MCF-7 cell lines. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was examined as loading control. (B, C) Effect of 4D3 (1 µg/ml) on growth inhibition by PTX (10 or 20 nM) in comparison with that of 2C1 (1 µg/ml) in MDA-468 cells (B) and MCF-

7 cells (C). (D) The intracellular PTX concentration was measured by enzyme-linked immunosorbent assay (ELISA) in cells treated with PTX (20 nM) exposed to 4D3 (1 µg/ml) or none. (E) Induction of apoptosis by PTX (20 nM) with or without exposure to 4D3 (1 µg/ml). Error bar, standard deviation (SD) from three independent examinations.

Figure 3

Figure 3

The combined effects of paclitaxel (PTX) and 4D3 in in vivo (A) Subcutaneous tumors of MDA-468 and MCF-7 cells in nude mice were treated with PTX (10 mg/kg BW) and/or 4D3 (1 mg/kg BW) on Day 1, 3, and 7. Tumor weights 4 weeks after inoculation were displayed. (B) Lung metastasis resulted by caudal inoculation of MDA-468 and MCF-7 cells in nude mice that were treated with PTX (10 mg/kg BW) and/or 4D3 (1 mg/kg BW) on Day 1, 3, and 7. Lung weights, 4 weeks after inoculation, were displayed. (C) mRNA expression of markers for mouse stromal cells in the subcutaneous tumors were examined by reverse transcriptase polymerase chain reaction (RT-PCR). Batf2, M1 macrophage; Fizz, M2 macrophage; CD73, mesenchymal stem cell. (D) mRNA expression of human stemness-associated genes, CD133 and CD44, were examined by RT-PCR. (E) Intratumoral pH of the subcutaneous tumors was measured. (F) mRNA expression of human stemness-associated genes were examined in IDC cells cultured in media adjustment of the pH to 7.4 or 7.0. Error bar, standard deviation (SD) from 5 mice. β -actin (BACT) was examined as a loading control.

Figure 4

Figure 4

The combined effects of paclitaxel (PTX) and 4D3 with tamoxifen (TAM) or bisphosphonate (BP) in in vivo (A, B) Lung tumors of MDA-468 cells (A) and MCF-7 cells (B) in nude mice were treated with PTX (10 mg/kg BW) and/or TAM (500 mg/mouse) and/or 4D3 (1 mg/kg BW) on Day 1, 3, and 7. Fluorescence intensities of tumors 4 weeks after inoculation were displayed. (C, D) Survivals of mice examined in panels A and B. (E, F) Intratibial tumors of MDA-468 cells (E) and MCF-7 cells (F) in nude mice were

treated with PTX (10 mg/kg BW) and/or BP (zoledronic acid, 100 µg/kg BW) and/or 4D3 (1 mg/kg BW) on Day 1, 3, and 7. Fluorescence intensities of tumors 4 weeks after inoculation were displayed. Error bar, standard deviation (SD) from five mice.

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