Suppression of the vitamin D metabolizing enzyme CYP24A1 provides increased sensitivity to chemotherapeutic drugs in breast cancer

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Abstract. Vitamin D is an essential nutrient for the human body not only for the metabolism of calcium but also for homeostasis. Vitamin D contributes to cell fate decisions, including cell proliferation, differentiation and viability. Accumulated epidemiological data suggest a relationship between vitamin D deficiency and carcinogenesis in numerous organs. Furthermore, it is known that the expression of the vitamin D metabolizing enzyme, cytochrome P450 family 24 subtype A1 (CYP24A1), is increased in different types of human malignancy including breast carcinoma. However, the pathological relevance of elevated CYP24A1 expression level requires further clarification. In the present study, it was demonstrated that CYP24A1 promoted the oncogenic property of breast carcinoma cells. Consistent with previous reports, it was demonstrated that the expression of CYP24A1 was elevated in invasive breast carcinoma and significantly decreased the overall survival of patients with invasive breast carcinoma. Importantly, suppression of CYP24A1 expression significantly enhanced cell death sensitivity to two anticancer drugs with pharmacologically different modes of action, cisplatin and gefitinib. The results of the present study suggest the possibility of CYP24A1-inhibiting therapy as a novel therapy in breast cancer with overexpression of CYP24A1.

Introduction

Vitamin D is an essential nutrient for the human body and is not only crucial for regulation of calcium metabolism but also serves an important role in homeostasis (1-4). 1,25-dihydroxyvitamin D ($1\alpha,25(OH)_2D_3$), also known as calcitriol, is the active form of vitamin D. Previous studies have reported that $1\alpha,25(OH)_2D_3$ is a ligand of nuclear vitamin D receptor (VDR), that contributes to numerous processes in the body, including cell proliferation, differentiation and cell viability (5-7). $1\alpha,25(OH)_2D_3$ can act protectively against cancer by promoting apoptosis (8), and the relationships between vitamin D deficiency and numerous types of cancer, such as colorectal cancer and prostate cancer have been reported in previous studies (9-11). Furthermore, it has been previously shown that supplementation of vitamin D suppresses carcinogenesis in numerous organs, such as breast, prostate, colorectal, and head and neck cancer (12). However, the underlying mechanism linking tumorigenicity and cellular vitamin D status remains unknown.

The bioavailability of vitamin D is regulated by a coordinated balance between $1\alpha,25(OH)_2D_3$ biosynthesis and catabolism, and causally determines cellular responses to vitamin D (1-3). The vitamin D metabolizing enzyme cytochrome P450 family 24 subtype A1 (CYP24A1) contributes to the inactivation of $1\alpha,25(OH)_2D_3$ by converting it to rapidly excreted derivatives (3,5). This enzymatic activity restricts the access of $1\alpha,25(OH)_2D_3$ to the transcriptional machinery and limits vitamin D signaling within cells (5). It has been previously reported that CYP24A1 expression is elevated in certain types of tumor cells, such as breast, prostate, colorectal, and head and neck cancers and that numerous types of cancer cells contain inactive vitamin D metabolites such as $1\alpha,24,25\text{-}(OH)_3D_3$ and 24-oxo-$1\alpha,25\text{-}(OH)_2D_3$ (13,14).

Previous studies reported that CYP24A1 has an oncogenic activity in breast cancer (15); however, the clinical relevance of vitamin D depletion induced by CYP24A1 in breast cancer remains to be clarified. In the present study, the expression of CYP24A1 in surgically resected breast tumor specimens and the effect of CYP24A1 expression on carcinogenesis in breast carcinoma cells was evaluated.

Materials and methods

Patients and specimens. Tissue specimens from 136 cases of breast cancer collected from Sapporo Medical University Hospital (Sapporo, Japan) during surgical resection from

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2011-2014 were used in the present study. Data were also collected from the pathology file of Sapporo Medical University Hospital. The mean age of the patients was 59.3 years (range, 26-92 years). Histological type was based on the World Health Organization (WHO) classification of tumors of the breast (5th edition) (16). For intrinsic subtype classification, surrogate molecular breast cancer classification based on immunohistochemical assessment of the estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and Ki-67 biomarkers was used according to the WHO classification of tumors (5th edition) (16). All of the 136 cases were staged according to the Union for International Cancer Control stage classification (7th edition) in the WHO classification of tumors (5th edition) (Table I) (16). In the staging process of breast tumors, we categorized tumors using parameters such as pT factor, which is defined as pathological status of primary tumor, and pN factor, which is defined as pathological status of lymph node involvement (16). The present study was approved by the Ethics Committee (approval no. 4-1-44) and Institutional Review Board (study no. 312-230) of Sapporo Medical University (Sapporo, Japan). The Ethics Committee waived the requirement to obtain written informed consent from the patients for the use of human tissues owing to the retrospective nature of the study. The research was performed in accordance with the Declaration of Helsinki. The researchers involved in this study had no access to information that could identify individual participants during or after data collection.

Immunohistochemical staining. Tissue sections were fixed with 10%-buffered formalin overnight at room temperature. Fixed tissues were embedded in paraffin and embedded sections were cut at 5 μm thickness. Tissue sections were then deparaffinized in xylene, rehydrated using a decreasing ethanol series and incubated in 3% H_2O_2 for 10 min to block endogenous peroxidase activity. After antigen retrieval by microwave heating (95˚C for 30 min) in 10 mmol/l Tris and 1 mmol/l EDTA buffer, the sections were incubated overnight at 4˚C with primary monoclonal antibodies against CYP24A1 (1:100; cat. no. sc-365700; Santa Cruz Biotechnology, Inc.). The sections were then incubated with EnVision (Dako; Agilent Technologies, Inc.) for 30 min at room temperature, and 3,3'-diaminobenzidine tetrahydrochloride (Dako; Agilent Technologies, Inc.) was added as the chromogen. Hematoxylin was used for counterstaining at room temperature for 3 min. Analysis of immunohistochemical staining positivity was performed using a light microscope, based on the staining intensity and the percentage of positive cells. The intensity scores of staining were set as follows: 3+, strong; 2+, moderate; 1+, weak; and 0, negative. The observers were blinded to the clinical data during the evaluation. Consensus was reached by discussion of discordant cases.

Cell culture and transfection. The ER positive, breast cancer MCF7 cell line, was purchased from a local distributor (Summit Pharmaceuticals International Corporation) of the American Type Culture Collection. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, MilliporeSigma) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) and 5% streptomycin (MilliporeSigma). The cells were transfected with different types of CYP24A1-specific small-hairpin RNA (shRNA)-expressing lentivirus plasmids according to manufacturer's instruction provided (MISSION® shRNA Plasmid DNA; MilliporeSigma). Transfection was performed with 5 μg shRNA plasmid, using FuGENE6 (Roche Diagnostics) to generate stable transfectants. Cells were incubated with plasmid for 48 h at 37˚C in a humidified 5% CO_2 atmosphere. The shRNAs used were as follows: CYP24A1 shRNA #1296 (shRNA clone ID: NM_ 000782.2-1296s1c1, MilliporeSigma) with the plasmid sequence, 5'-CCGGGCAGATTTCCTTTG TGACATTCTCGAGAATGTCACAAGGAAATCTGCTT TTTG-3' and CYP24A1 shRNA #1016 (shRNA clone ID: NM_000782.2-1016s1c1, MilliporeSigma) with the plasmid sequence, 5'-CCGGCGAACTGAACAAATGGTCGTTCT CGAGAACGACCATTTGTTCAGTTCGTTTTTG-3'. Transfected clones were selected using 1.5 μg/ml puromycin (MilliporeSigma). Drug-resistant clones were selected after ≥14 days of selection and screened for CYP24A1 expression to measure their CYP24A1 RNA expressions using reverse transcription (RT)-PCR analysis. Following screening, the MCF7 cell transfectants, CYP24A1 shRNA #1 and CYP24A1 shRNA #7 were used in subsequent experiments. We have previously reported that the process of transfection with CYP24A1 scramble shRNA did not affect the cell phenotype (15). Therefore, wild-type MCF7 cells were used as the control in the present study.

Semi-quantitative RT-PCR analysis. Total RNA of wild-type MCF7 cells and their transfectants was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and subsequent RT-PCR was performed using the Superscript II Reverse Transcriptase kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's protocols. Samples were incubated at 42˚C for 50 min followed by incubation at 70˚C for 15 min. The complementary DNA was then mixed with the primers as follows: CYP24A1 forward (F), 5'-GCAGCCTAGTGC AGATTTCC-3' and reverse (R), 5'-ACCAGGGTGCCTGAG TGTAG-3'; and GAPDH F, 5'-GTCTCCTCTGACTTCAAC AGCG-3' and R, 5'-ACCACCCTGTTGCTGTAGCCAA-3', as well as 0.5 U of Taq DNA polymerase (Takara Bio, Inc.) to amplify the genes of interest. The thermocycling conditions used were as follows: 40 cycles at 96˚C for 30 sec, 30 sec at 55˚C, and 1 min at 72˚C, followed by a final elongation stage at 72˚C for 7 min. RNA expression was examined by loading on 1.5% agarose gels and visualized by ethidium bromide staining. As a loading control, 50 ng of 200 bp DNA ladder (Takara Bio, Inc.) was used. Finally, RNA expression levels were semi-quantified using ImageJ software (version 1.52; National Institutes of Health) and normalization to GAPDH expression.

Immunofluorescent assay. Cells were seeded in 35 mm dishes (10,000 cells/dish) containing 15 mm glass coverslips (AGC Techno Glass Co., Ltd.) and incubated with DMEM containing 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.). Glass coverslips were pre-coated with 1:1 rat tail collagen overnight at room temperature (Invitrogen; Thermo Fisher Scientific, Inc). The cells on the coverslips were fixed at 20˚C for 10 min with a fixing solution (acetone:ethanol, 1:1). The cells were

Table I. Association between CYP24A1 expression examined by immunohistochemistry and certain clinicopathological parameters.

Parameter	Total number of cases	CYP24A1		P-value
		Positive	Negative	
Histology				0.0067
DCIS	29	17	12	
IDC	103	86	17	
ILC	3	3	0	
Paget	1	0	1	
Primary tumor status				0.1070
pT1a	4	3	1	
pT1b	13	9	4	
pT1c	49	41	8	
pT1mi	4	3	1	
pT2	39	34	5	
pT3	6	4	2	
pT4b	1	1	0	
pTis	20	11	9	
Lymph node involvement				0.3000
pN0	100	76	24	
pN1a	23	19	4	
pN1c	2	2	0	
pN1mi	2	2	0	
pN2a	6	6	0	
pN3a	3	1	2	
Stage				0.0294
0	20	11	9	
IA	52	40	12	
IB	1	1	0	
IIA	40	35	5	
IIB	14	12	2	
IIIA	6	6	0	
IIIC	3	1	2	
Subtype				0.0047
Luminal A	46	30	16	
Luminal B	68	57	11	
HER2	7	4	3	
Basal type	15	15	0	
Age				0.4320
20-39	7	6	1	
40-59	56	47	9	
60-79	66	48	18	
80-99	7	5	2	

Samples were defined as positive for CYP24A1 if staining intensity 3+ was >50%. pT and pN confirmed by pathological examination were staged according to the Union for International Cancer Control stage classification (7th edition) in the WHO classification of tumors (5th edition) (16). CYP24A1, cytochrome P450 family 24 subtype A1; DCIS, Ductal carcinoma *in situ*; IDC, Invasive ductal carcinoma; ILC, Invasive lobular carcinoma; Paget, Paget's disease of the breast; pT, pathological status of primary tumor; pN, pathological status of lymph node involvement.

incubated with a primary monoclonal anti-CYP24A1 antibody (1:100, cat. no. sc-365700, Santa Cruz Biotechnology, Inc.) at 4˚C overnight, and then treated with Alexa Fluor 488 (green)-conjugated anti-rabbit IgG (1:200, cat. no. A-11008,

Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. The nuclei in the cells were counterstained using 4',6-diamidino-2-phenylindole at room temperature for 5 min (MilliporeSigma). The samples were imaged using an epifluorescence microscope (Olympus Corporation).

Treatment of cells. To evaluate cell viability, cells were seeded in 12-well dishes (40,000 cells/well) and the cells were counted by manual cell counting using trypan blue dye exclusion test (cells were stained with trypan blue at room temperature for 1 min) in a time and dose-dependent manner (dependent on the treatment conditions) using a light microscope (Olympus). To assess cell viability under different levels of cell stress, cells were treated with H_2O_2 (0, 25, 50 or 75 μM) for 4, 8 and 12 h to induce oxidative stress at 37˚C in a humidified, 5% CO_2 atmosphere. For the assessment of cell proliferation with and without treatment with vitamin D (1 μM, MilliporeSigma) and/or ketoconazole (2 μM, MilliporeSigma), cells were seeded in 12-well dishes (1,000 cells/well). The cells were treated with 1 μM vitamin D and 2 μM ketoconazole and incubated for 48 h at 37˚C in a humidified, 5% CO_2 atmosphere.

For the assessment of drug sensitivity, cells were seeded in 96-well plates (5,000 cells/well) and treated with cisplatin (0-100 nM, Adipogen AG) and gefitinib (0-100 nM; Cayman Chemical Company). The viability of cells treated with cisplatin was assessed every 24 h until 96 h of incubation and the viability of cells treated with gefitinib was assessed after 48 h. Cell viability was analyzed using a Cell Counting Kit-8 assay kit (Dojindo Laboratories, Inc.) according to the manufacturer's protocols. Absorbance at a wavelength of 450 nm was quantified using a Varioskan™ LUX microplate reader (Thermo Fisher Scientific, Inc.).

Immunocytochemistry of cell blocks. Cells were treated with H_2O_2 (0, 100 or 750 μM) for 24 h at 37˚C in a humidified, 5% CO_2 atmosphere to induce oxidative stress. Cells were harvested from culture dishes using a cell lifter and then collected by centrifugation at 300 x g for 3 min at room temperature. The collected cells were solidified using 10% agarose gel and fixed in 10% buffered formalin overnight at 4˚C. These tissues were paraffin-embedded and the tissue sections were cut at 5 μm thickness. Immunostaining was performed using primary antibodies against cleaved caspase-3 (1:50, cat. no. 9664; Cell Signaling Technology, Inc.) and Ki-67 (MIB-1 clone; 1:200, cat. no. AM297-5M, BioGenex Laboratories) at room temperature for 1 h. The sections were then incubated with EnVision (1:1, cat. no. K400311, Dako; Agilent Technologies, Inc.) at room temperature for 30 min. After washing with PBS, 3,3'-diaminobenzidine tetrahydrochloride (1:1, cat. no. GE001, Dako; Agilent Technologies, Inc.) was added as the chromogen at room temperature for 5 min.

Colony formation. Cells were seeded in 12-well plates (2,500 cells/well). After incubation of cells for 7 days at 37˚C in a humidified 5% CO_2 atmosphere, the cells were fixed using 10% buffered formalin for 15 min at room temperature and stained using 0.04% crystal violet for 15 min at room temperature. Cell clusters >50 μm in diameter were defined as positive; colonies were counted using phase-contrast microscopy (Olympus Corporation) under low magnification (x100) and were quantified using ImageJ software (version 1.52; National Institutes of Health).

Statistical analysis. At least 3 independent experiments were performed for each analysis and all data were presented as mean ± standard deviations. All data from each experiment were analyzed with either unpaired Student's t-test, Fisher's exact test or the Kruskal-Wallis test to determine significance. Bonferroni's post-hoc test was used where appropriate. Survival curves were constructed, and the Kaplan-Meier method and log-rank test were used to calculate the survival rate. R (version 4.0.3; RStudio, Inc.) was used for all statistical analyses. $P<0.05$ was considered to indicate a statistically significant difference.

Results

CYP24A1 is expressed in primary breast neoplasia. Previous studies reported that CYP24A1 is highly expressed in different types of cancer (13-15). In the present study, the correlations between CYP24A1 expression and the clinicopathological parameters of breast cancer were evaluated using immunohistochemistry (Table I and Fig. 1A-C). CYP24A1 was strongly expressed but its expression area was limited in normal ductal and acinar cells (data not shown). In non-invasive breast carcinoma, samples were positive for CYP24A1 expression 58.6% (17/29) of the cases. Consistent with previous reports, the CYP24A1-positive rates were 83.5% (86/103) and 100% (3/3) in invasive ductal carcinoma and invasive lobular carcinoma, respectively (P=0.0067, Fisher's exact test). No significant association of CYP24A1 expression with primary tumor status and lymph node involvement was demonstrated; however, tumor stage was significantly positively associated with a high expression level of CYP24A1 (P=0.0294, Kruskal-Wallis test).

Based on immunohistochemical assessment of the biomarkers ER, PR, HER2 and Ki-67, breast tumors can be classified into four major immunohistochemically surrogate intrinsic subtypes as follows: Luminal A (ER+, PR+/-, HER2-), Luminal B (ER+, PR+/-, HER2-, higher Ki-67 expression), HER2 (ER-, PR-, HER2+) and basal (ER-, PR-, HER2-) (16). Although some are overlapping, the prognosis of breast cancer patients has been reported to become worse in the order of luminal A, luminal B, HER2-overespressed type, and basal subtype (15-19). In the present study, intrinsic surrogate subtype was associated with CYP24A1 expression, with the expression of CYP24A1 being significantly higher in subtypes with poor prognosis. Indeed, a significant increase in the expression of CYP24A1 was noted in hormone receptor negative cancer (P=0.0047, Kruskal-Wallis test).

The cases were divided into two groups based on the expression of CYP24A1 assessed by staining intensity and area. Specimens containing >50% area with staining intensity 3+ were defined as positive, and specimens containing ≤50% area with staining intensity 3+ were defined as negative. Kaplan-Meier survival curves demonstrated that the overall survival rate in the CYP24A1-positive group was markedly lower than that in the CYP24A negative group when compared in whole specimens (Fig. 1D). However, a significant association between the CYP24A1 positivity and overall survival rate was demonstrated in invasive breast carcinoma (P=0.0266;

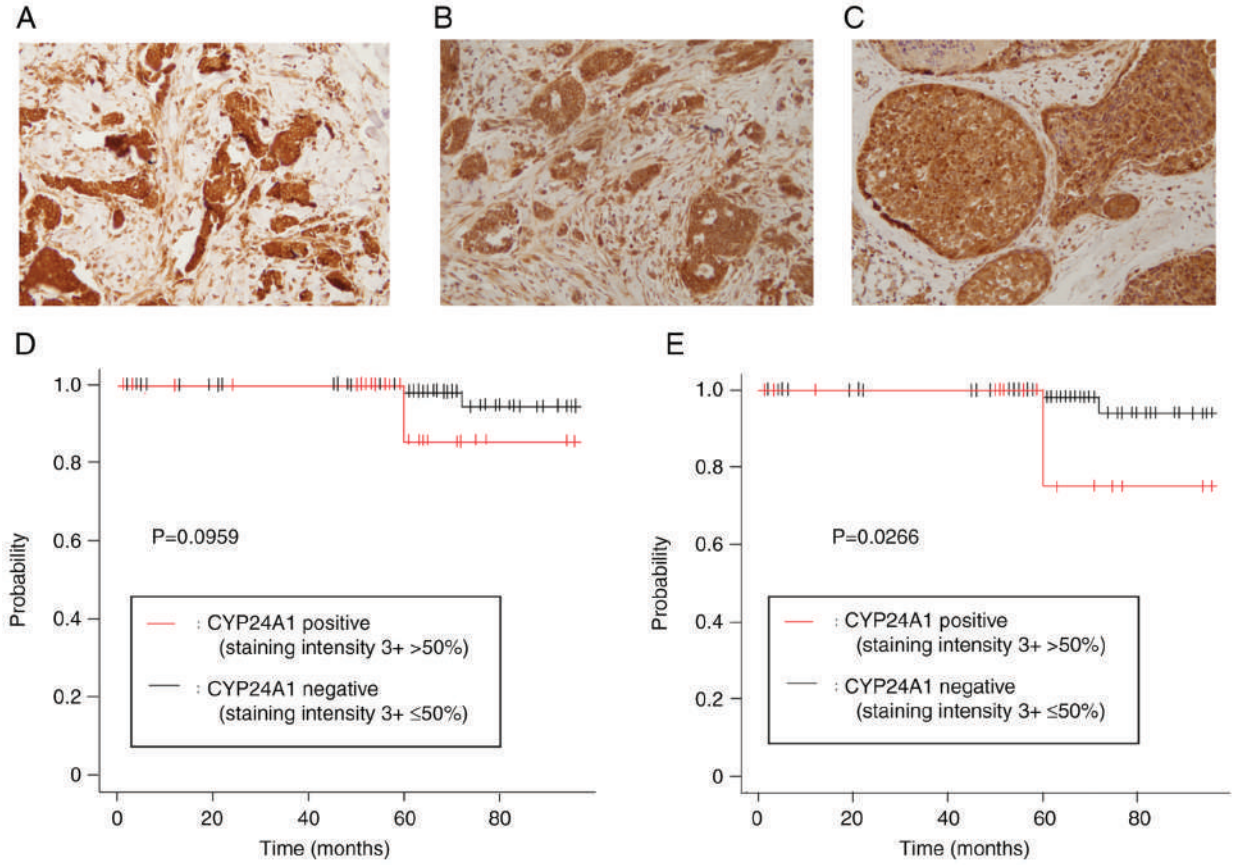

Figure 1. Expression of CYP24A1 in primary breast neoplasia. (A-C) Representative cases of CYP24A1 positivity (staining intensity 3+ >50%; magnification, x200). (D,E) Kaplan-Meier analysis of overall survival of patients with CYP24A1-positive and CYP24A1-negative specimens in (D) whole breast tumor and (E) invasive breast cancer. CYP24A1, cytochrome P450 family 24 subtype A1.

Fig. 1E). These results were consistent with results of previous studies which suggested a possible oncogenic effect of CYP24A1 in the growth of a breast neoplasm (13-15).

Establishment of CYP24A1 knockdown cells. For the establishment of MCF7 cells with suppressed CYP24A1 expression, cells were transfected with shRNAs against CYP24A1 with two different sequences and the cell lines were denoted as CYP24A1 shRNA #1 and CYP24A1 shRNA #7. An immunofluorescent assay demonstrated that the expression of CYP24A1 protein was markedly suppressed in both cell lines (Fig. 2A). RT-PCR analysis demonstrated that CYP24A1 RNA expression levels were markedly reduced in CYP24A1 shRNA #7 (~75%) and CYP24A1 shRNA #1 cells (~50%) (Fig. 2B).

Effect of CYP24A1 suppression on cell viability. To evaluate the effect of CYP24A1 knockdown on cell viability, MCF7 cells were cultured for 12 h with and without oxidative stress, induced using H_2O_2 (Fig. 3A). In the absence of H_2O_2, no difference in cell viability was demonstrated among all cell groups. The viability of CYP24A1 shRNA #7 cells cultured with H_2O_2 significantly decreased in a dose and time-dependent manner (Fig. 3B and C). However, there was no significant difference in the viability of CYP24A1 shRNA #1 cells cultured with and without H_2O_2.

Cells were treated with vitamin D and ketoconazole, a broad-spectrum inhibitor of CYP24A1, and a manual cell count was performed after 48 h. Although ER-positive cells such as MCF7 cells are known to express higher levels of VDR than the levels in ER-negative cells (1,16), vitamin D only demonstrated a marked decreased in the viability of CYP24A1 shRNA #7 cells. There was no marked difference in cell viability when ketoconazole was added (Fig. 3D).

Effect of CYP24A1 suppression on apoptosis. Cell blocks from cultured cells were established to assess cell death sensitivity under cell stress conditions and the number of apoptotic bodies were manually counted (Fig. 4A). Apoptosis was markedly increased in CYP24A1 knockdown cells. The number of apoptotic cells markedly increased in cells with suppression of CYP24A1 expression when a moderate level of oxidative stress (100 μM H_2O_2) was added. In controls, the number of apoptotic cells only increased with a higher level of oxidative stress (750 μM H_2O_2) (Fig. 4B). These results suggested that cells with suppression of CYP24A1 expression had a higher cell death sensitivity to a cell stressor.

Immunohistochemistry was performed using an antibody against cleaved caspase-3 to evaluate the effects of altered CYP24A1 expression on apoptosis (Fig. 4C). The proportion of cleaved caspase-3-positive cells was significantly increased in CYP24A1 knockdown cells treated with H_2O_2 (Fig. 4D).

Effect of CYP24A1 suppression on colony-forming efficacy. To evaluate the effect of CYP24A1 knockdown on two-dimensional tumorigenicity with and without vitamin D and ketoconazole treatment, a colony formation assay was

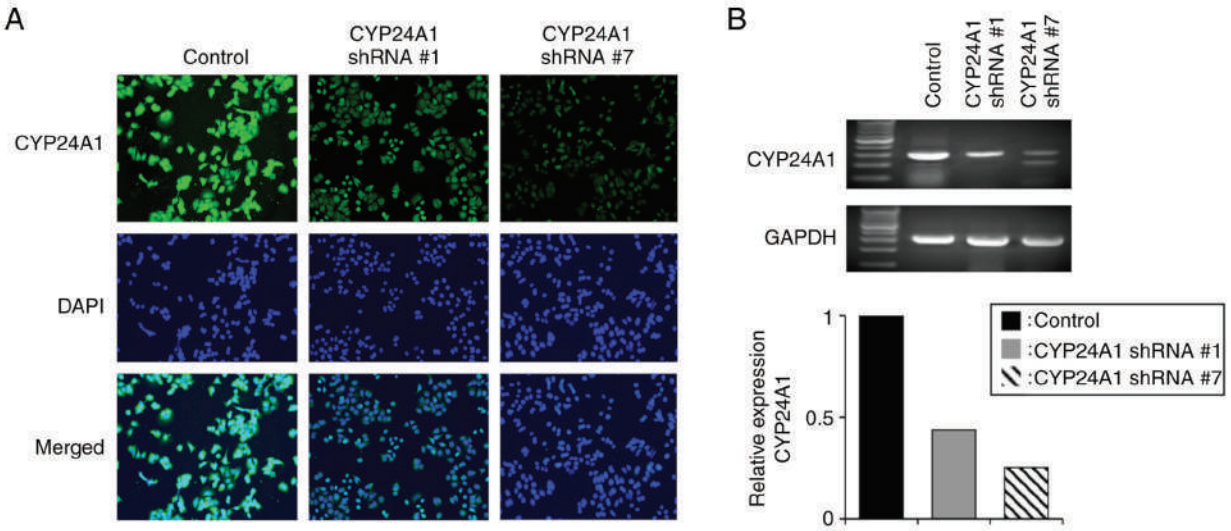

Figure 2. Establishment of CYP24A1 knockdown MCF7 cells. (A) Immunofluorescent assay for CYP24A1 (magnification, x100). (B) Reverse transcription-PCR analysis of CYP24A1 (upper panel) and its semi-quantitative analysis (bottom panel) relative to control cells. CYP24A1, cytochrome P450 family 24 subtype A1; shRNA, short-hairpin RNA.

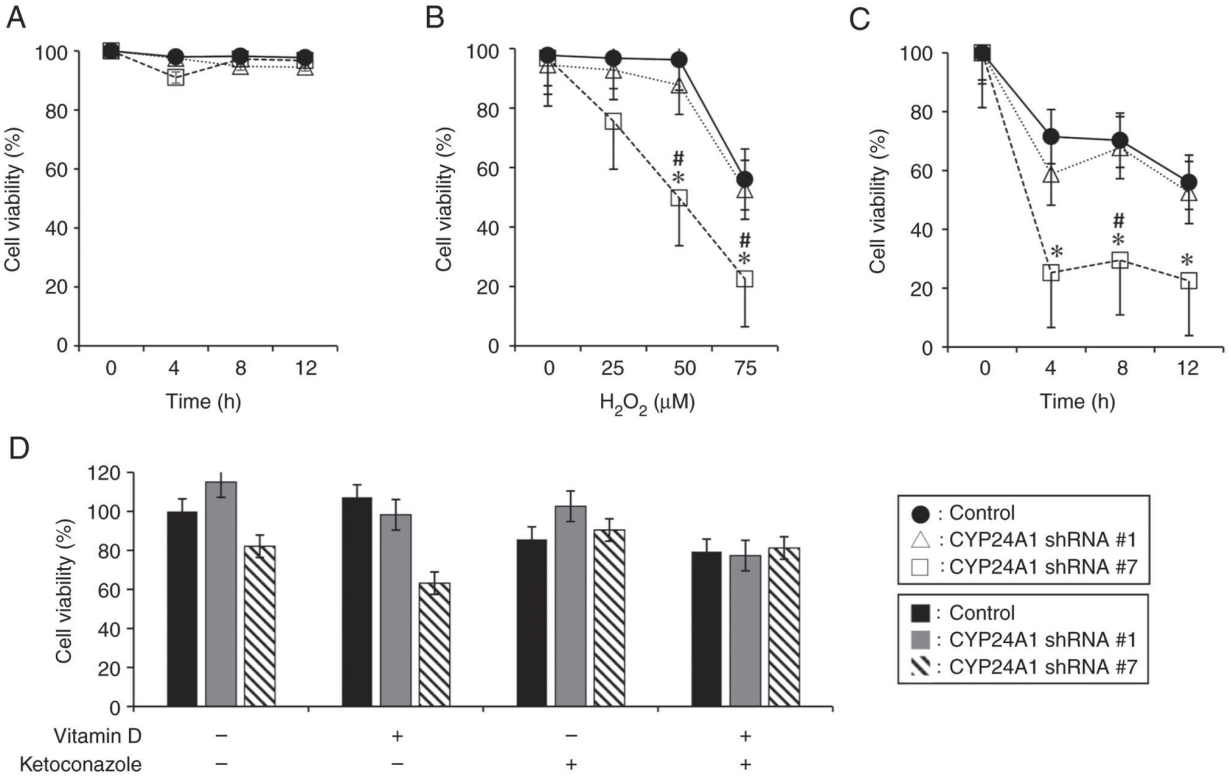

Figure 3. Effect of suppression of CYP24A1 expression on viability of MCF7 cells. (A) Cell viability without oxidative stress. (B) Cell viability with various levels of oxidative stress induced using H_2O_2 (25-75 μM). (C) Cell viability in a time-dependent manner with oxidative stress induced using H_2O_2 (75 μM). (D) Quantitative analysis of the viability of MCF7 cells with CYP24A1 suppression in the presence and absence of vitamin D (1 μM) and ketoconazole (2 μM). *P<0.05 vs. control; #P<0.05 vs. CYP24A1 shRNA #1. CYP24A1, cytochrome P450 family 24 subtype A1; shRNA, short-hairpin RNA.

performed (Fig. 5). Compared with the colony-forming ability of the control cells without treatment, this ability was markedly suppressed in both CYP24A1 shRNA #1 and shRNA #7 cells when untreated. Furthermore, colony formation efficacy was suppressed more in CYP24A1 shRNA #7 cells compared with that by CYP24A1 shRNA #1 cells. In the presence of vitamin D, the area of the colonies was markedly decreased in all cell groups. The results suggested that cellular vitamin D status effected colony formation efficacy. However, ketoconazole did not affect colony formation compared with the untreated groups.

Effect of CYP24A1 suppression on cell death sensitivity to anticancer drugs. To evaluate cell sensitivity to anticancer

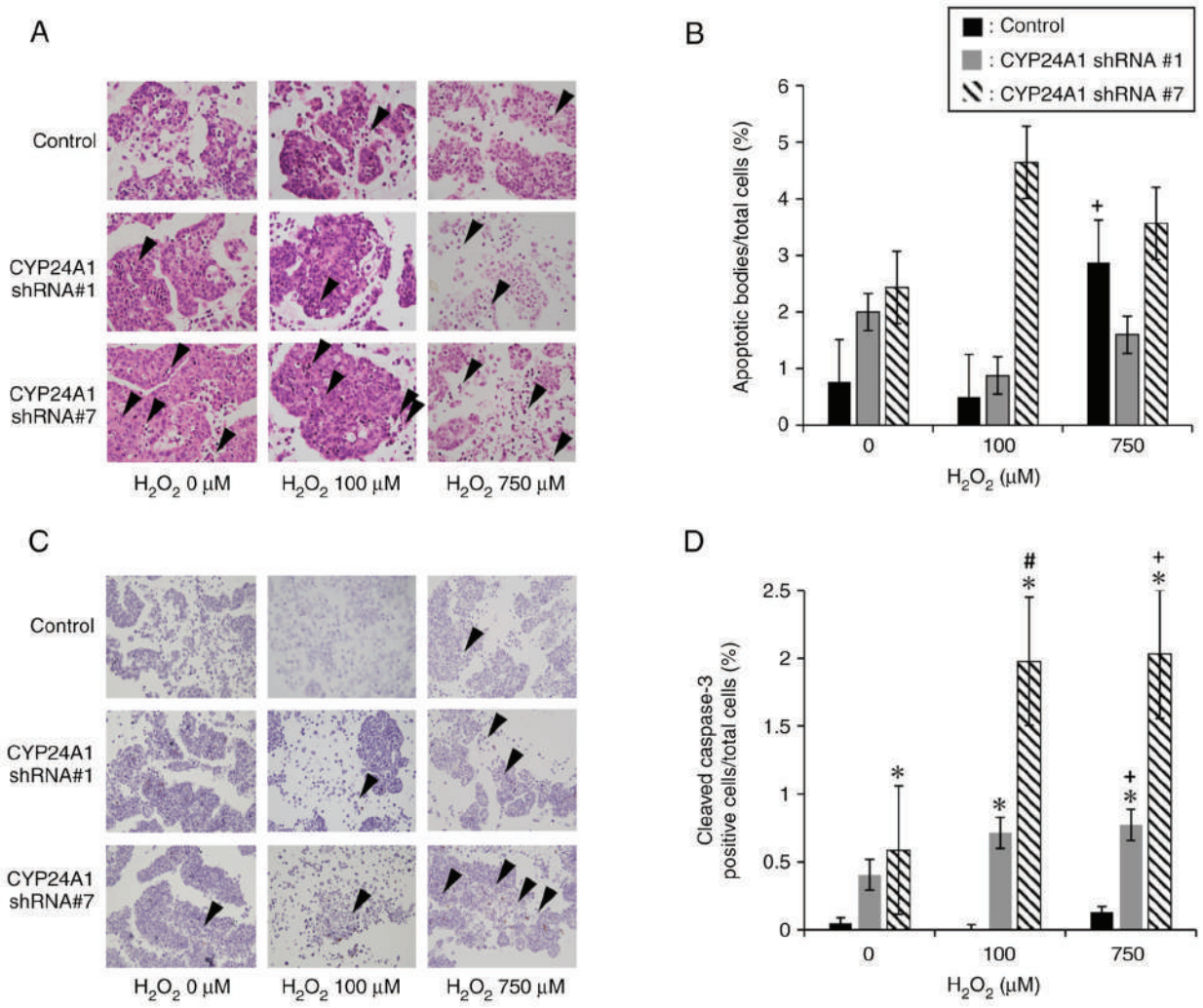

Figure 4. Effect of CYP24A1 suppression on cell apoptosis. Apoptosis was evaluated using cell blocks cultured with different doses of H_2O_2 (100 and 750 μM). (A) Representative images of manual cell counting of apoptotic bodies (arrow heads indicated apoptotic bodies; magnification, x200). (B) Quantitative analysis of the manual cell counting of apoptotic bodies. (C) Representative images of immunocytochemistry for cleaved caspase-3 (arrow heads indicated cleaved caspase-3 positive cells; magnification, x100). (D) Quantitative analysis of immunocytochemistry of cleaved caspase-3. *P<0.05 vs. control; $^{\#}$P<0.05 vs. CYP24A1 shRNA #1; $^{+}$P<0.05 vs. untreated. CYP24A1, cytochrome P450 family 24 subtype A1; shRNA, short-hairpin RNA.

drugs with different pharmacological mechanisms (cisplatin and gefitinib), cells were cultured with each drug and cell viability was analyzed (Fig. 6). Reduced expression of CYP24A1 significantly enhanced cell death sensitivity to both cisplatin and gefitinib, compared with the control.

Discussion

The present study demonstrated for the first time that increased expression of CYP24A1 leads to a decrease in the overall survival of patients with invasive primary breast carcinoma. Furthermore, it was demonstrated that suppression of CYP24A1 expression inhibited the oncogenic activity of breast carcinoma cells and enhanced cell sensitivity to anti-cancer drugs with different pharmacologic activities. These results suggested CYP24A1 as a possible therapeutic target in CYP24A1-expressing breast malignancy.

The prognosis and treatment response differ among the intrinsic surrogate subtypes of breast cancer, with the basal subtype having the worst prognosis and Luminal A subtype having the best prognosis (17-20). Previous studies reported that ER+ breast cancer cell lines were more sensitive to the effects of calcitriol. In the present study, the protein expression level of CYP24A1 was higher in the intrinsic subtypes reported to be associated with a poor prognosis, and particularly in the basal subtype (Table I), which suggested that CYP24A1 was a possible prognostic marker in breast cancer. This hypothesis was supported by the overall survival rate of patients with a strong expression of CYP24A1 in invasive ductal carcinoma, which was significantly lower compared with that in patients with only moderate or no expression of CYP24A1 (Fig. 1).

Previous studies have reported that $1\alpha,25(OH)_2D_3$ serves as a ligand for VDR. Vitamin D suppresses carcinogenesis and serves an important role in tissue homeostasis by the regulation of the expression of genes affecting cell proliferation, differentiation and apoptosis (15,21-23). $1\alpha,25(OH)_2D_3$ serves an important role in the promotion of apoptosis by the regulation of calcium signaling through calcium channels linked to the membrane VDR (1-3,23). In the process of apoptosis, the concentration of intracellular calcium increases and

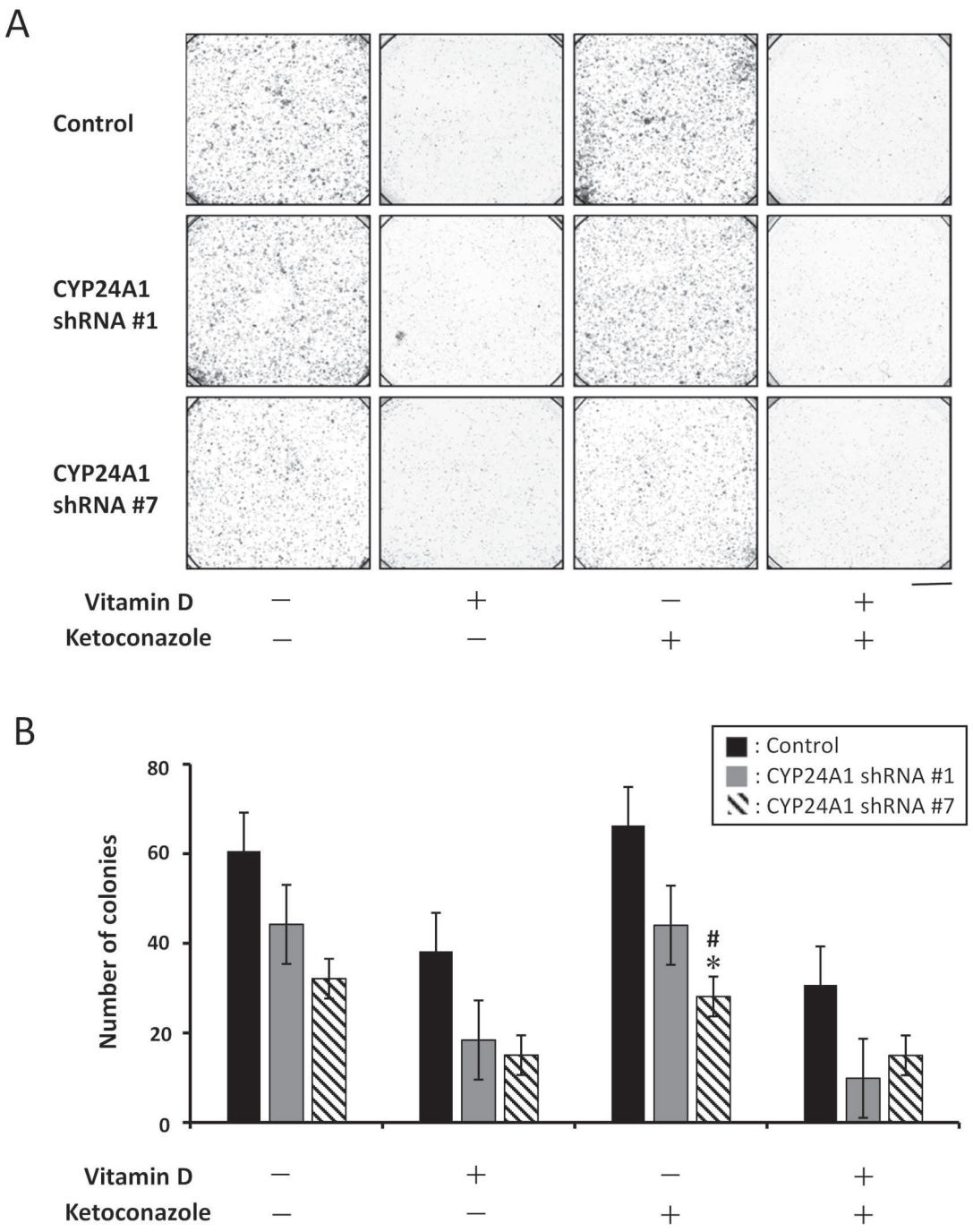

Figure 5. Effect of CYP24A1 suppression on colony forming efficacy of MCF7 cells with and without vitamin D (1 μM) and ketoconazole (2 μM). (A) Representative images of colony formation. Scale bar=1 mm. (B) Quantitative analysis of colony-forming efficacy. *P<0.05 vs. control; #P<0.05 vs. CYP24A1 shRNA #1. CYP24A1, cytochrome P450 family 24 subtype A1; shRNA, short-hairpin RNA.

interacts with molecular calcium-dependent targets within cells, including calcium-activated apoptotic effectors (1-3,15,20). CYP24A1 converts $1\alpha,25(OH)_2D_3$ into rapidly excreted inactive derivatives and restricts the access of $1\alpha,25(OH)_2D_3$ to the transcriptional machinery, which limits vitamin D signaling within cells (15,23). These reports suggest that CYP24A1 has as a desensitizing effect on apoptosis-inducing factors through calcium signaling mediated apoptotic inducers.

The present study demonstrated that suppression of CYP24A1 expression significantly increased apoptosis in breast tumor cells under different types of cell stressors such as oxidative stress mediated by H_2O_2 and chemotherapeutic drugs. The results of the present study demonstrated that supplementation of vitamin D markedly decreased cell viability and colony-forming efficacy (24); however, the effect was not statistically significant. Furthermore, the addition of ketoconazole did not affect the viability and colony-forming efficacy of MCF7 cells (25,26), although the suppression of CYP24A1 expression itself markedly decreased these effects (Fig. 5). These results suggested that CYP24A1 has an as-yet-unrecognized activity independent of vitamin D metabolism. It has been previously reported that CYP24A1

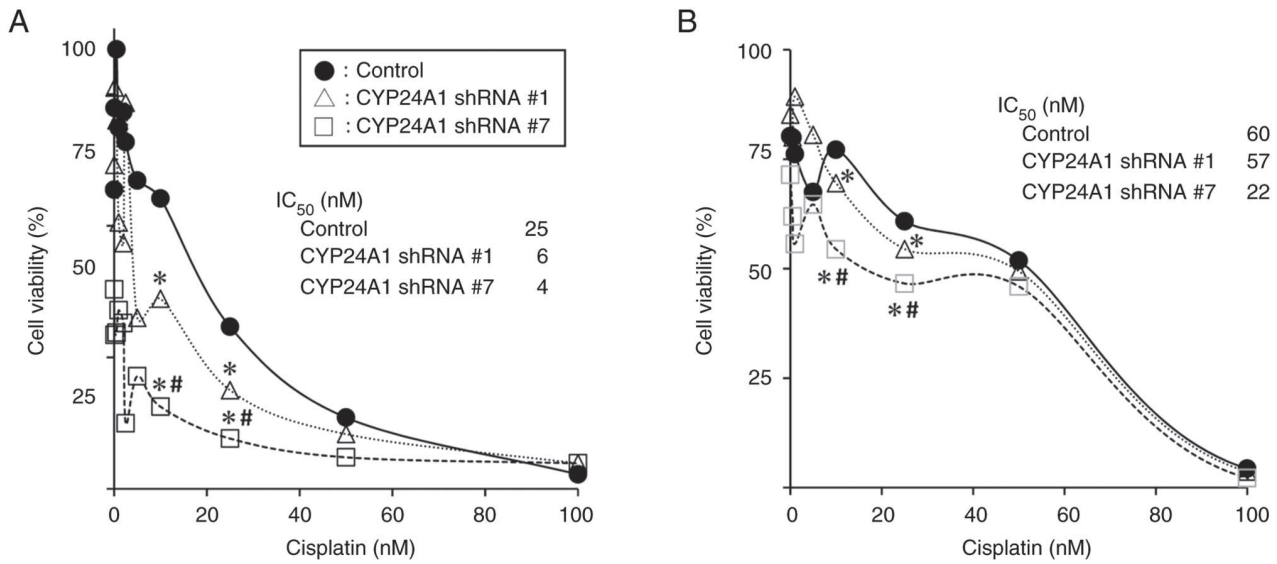

Figure 6. Effect of CYP24A1 suppression on cell death sensitivity to anticancer drugs. MCF7 cells were treated with different doses of (A) cisplatin and (B) gefitinib, and the IC_{50} was calculated. *P<0.05 vs. control; #P<0.05 vs. CYP24A1 shRNA #1. CYP24A1, cytochrome P450 family 24 subtype A1; shRNA, short-hairpin RNA.

expression is elevated in various types of tumors and correlates with poor prognosis (13-15). Therefore, a CYP24A1-specific inhibitor might be able to effectively inhibit the tumorigenicity of CYP24A1-expressing breast malignancy.

The results of the present study demonstrated that suppression of CYP24A1 expression in breast cancer cells increased cell sensitivity to two anticancer drugs with different pharmacological mechanisms. The first anticancer drug used was cisplatin, a chemotherapeutic drug that induces cell apoptosis in cancer cells by crosslinking with the purine bases on DNA which disrupts the DNA repair mechanism (27). The second anticancer drug was gefitinib, which is a tyrosine-kinase inhibitor used for of the treatment of numerous types of cancers including HER2-positive breast cancer (28). The results of the present study indicated that CYP24A1 enhanced cell death activity in response to cell death inducers with different mechanisms of action. Therefore, inhibition of CYP24A1 activity could be a possible therapeutic approach in breast malignancy.

A limitation of the present study is that animal experiments were not included and that only one type of breast cancer cell line (MCF7) was used. In our preliminary study, animal experiments were performed; however, the effects of CYP24A1 suppression on tumor growth were not statistically significant (data not shown). Furthermore, to evaluate the role of CYP24A1 in other cell lines with different expression levels of ER, PR and HER2, our preliminary study attempted to establish CYP24A1 knockdown cells with the T47D (Luminal A), ZR-75 (Luminal B) and SK-BR-3 (HER2) cell lines; however, none of the cells survived when CYP24A1 was knocked down using two different shRNA constructs. The cell line that was used in the present study, MCF7, is a good candidate for the evaluation of the effect of vitamin D on breast cancer cells as vitamin D deficiency is known to be associated with poor outcomes in patients with luminal-type breast cancer such as MCF7 cells (29). Furthermore, ER-positive cells have been reported to express higher levels of VDR compared with the levels in ER-negative cells (16,22) and the results of previous studies also showed that dietary intake of vitamin D reduces the risk of ER-positive breast cancer (30-32). Together with the results of our previously published study (15), it can be suggested that CYP24A1 is indispensable for the survival of those breast cancer cell lines. If CYP24A1 has the same effect on those breast tumor cells with different hormone receptor status as it has on MCF7 cells, CYP24A1 inhibiting therapy might have an even greater impact on those cells. Further studies using different cell lines with various expression levels of ER, PR and HER2 are needed.

The results of the present study demonstrated that high level expression of CYP24A1 in invasive breast cancer led to a significant decrease in the overall survival rate of patients with breast carcinoma. Furthermore, it was demonstrated that suppression of CYP24A1 expression *in vitro* decreased the tumorigenicity of breast carcinoma cells and increased cell sensitivity to differently acting anticancer drugs. In conclusion, the results of the present study suggest that CYP24A1 is a possible therapeutic target for breast malignancy with constitutive CYP24A1 expression.

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Availability of data and materials

The datasets generated and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

SK and MO substantially contributed the conception and design of this study. SK and YN performed the cell culture experiments and immunohistochemistry. AT, KT, DK, YO and KM performed histological examination of the breast cancer. SK, YN and MO confirm the authenticity of all the raw data obtained. SK and MO were major contributors to data analysis and interpretation of the data. SK and MO contributed to manuscript drafting and critical revisions on the intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was reviewed and approved by the Institutional Ethics Committee (approval no. 4-1-44) and Institutional Review Board (study no. 312-230) of Sapporo Medical University. Specimens of 136 cases of breast cancer collected by surgical resection from 2011-2014 were used in the present study. The Ethics Committee waived the requirement to obtain written informed consent from the patients for the use of human tissues owing to the retrospective nature of the study. The research was conducted in accordance with the Declaration of Helsinki. The researchers involved in this study had no access to information that could identify individual participants during or after data collection.

Patient consent to participate

Not applicable.

Competing interests

The authors declare that they have no conflicts of interests.

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