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CITED2 in breast carcinoma as a potent prognostic predictor associated with proliferation, migration and chemoresistance

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Key words

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CITED2 (Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2) is a member of the CITED family and is involved in various cellular functions during development and differentiation. Mounting evidence suggests the importance of CITED in the progression of human malignancies, but the significance of CITED2 protein has not yet been examined in breast carcinoma. Therefore, in the present study, we examined the clinical significance and the biological functions of CITED2 in breast carcinoma by immunohistochemistry and *in vitro* study. CITED2 immunoreactivity was detected in breast carcinoma tissues, and it was significantly higher compared to those in morphologically normal mammary glands. CITED2 immunoreactivity was significantly associated with stage, pathological T factor, lymph node metastasis, histological grade, HER2 and Ki-67, and inversely correlated with estrogen receptor. Moreover, the immunohistochemical CITED2 status was significantly associated with increased incidence of recurrence and breast cancer-specific death of the breast cancer patients, and multivariate analyses demonstrated CITED2 status as an independent worse prognostic factor for disease-free and breast cancer-specific survival. Subsequent *in vitro* experiments showed that CITED2 expression significantly increased proliferation activity and migration property in MCF-7and S KBR-3 breast carcinoma cells. Moreover, CITED2 caused chemoresistance to epirubicin and 5-fluorouracil, but not paclitaxel, in these cells, and it inhibited p53 accumulation after 5-fluorouracil treatment in MCF-7 cells. These results suggest that CITED2 plays important roles in the progression and chemoresistance of breast carcinoma and that CITED2 status is a potent prognostic factor in breast cancer patients.

Invasive breast cancer is generally regarded as a disease that metastasizes at an early stage, and adjuvant therapy, such as endocrine therapy and/or chemotherapy (epirubicin [EPI], 5-fluorouracil [5FU] and paclitaxel [PTX]), is frequently used after surgical treatment. However, some of these carcinomas acquire clinical resistance and recur despite the therapy. The recurrence rate was approximately 10% after 5-years of endocrine therapy in estrogen receptor (ER)-positive early breast cancer,[1] and results of 11 adjuvant chemotherapy trials revealed that 25% of the patients who received adjuvant chemotherapy developed distant recurrence.[2] Therefore, it is very important to evaluate biological markers in breast cancer patients to predict their cancer recurrence after surgery and to evaluate the need for additional therapies.

We previously compared gene expression profiles between recurrent and non-recurrent groups of ER-positive breast carcinoma patients after surgery, and identified 17 genes linked to the recurrence.[3] Among these, we are particularly interesting in CITED2 (Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2). CITED2 is a member of the CITED family and regulates various cellular functions during development and differentiation.[4] The CITED family includes three members, three of which are present in mammals, CITED1, 2 and 4,[5] and mounting evidence suggests the importance of CITED in the progression of breast carcinoma. For instance, CITED1 interacts with ER during normal development of mammary glands and CITED1 expression correlates with a good outcome in breast cancer.[6] Induction of HER2 expression by CITED1 has also been reported in breast carcinoma.[7] CITED4 expression is downregulated in breast carcinoma by DNA methylation and inhibits hypoxia-inducible factor 1α (HIF-1α) transactivation.[8,9] The association between CITED2 mRNA expression and prognosis of ER-positive human breast cancer has been examined by quantitative RT-PCR,[10,11] but the results are inconsistent and the significance of CITED2 remains unclear. This may be because the significance of CITED2 protein has not been examined in breast carcinoma tissues. Therefore, in this study, we examined CITED2 in breast carcinoma by immunohistochemistry and *in vitro* study to explore its clinical significance and biological functions.

Materials and Methods

Patients and tissues. For the present study, 109 specimens of invasive ductal carcinoma, not otherwise specified, were obtained from female Japanese patients who underwent surgical treatment from 2007 to 2008 in Tohoku University Hospital (Sendai, Japan). The patients were derived from a cohort of successive patients treated at Tohoku University Hospital, and review of the charts revealed that 52 patients received adjuvant chemotherapy, while 88 patients received adjuvant endocrine therapy after the surgery. In addition, we obtained 56 specimens of invasive ductal carcinoma, not otherwise classified, from female Japanese patients who underwent surgical treatment from 1995 to 1999 in Tohoku University Hospital (Sendai, Japan), as a second cohort for this study.

As shown in Table S1, the clinicopathological characteristics of the 109 breast carcinomas examined were not markedly different from those previously reported in breast carcinoma.[12] CITED2 immunoreactivity in non-neoplastic mammary glands was also available for examination in 80 out of the 109 cases examined in this study. Research protocols for the present study were approved by the Ethics Committee at Tohoku University School of Medicine.

Immunohistochemistry. Mouse monoclonal antibodies for CITED2 (LS-B243) and Ki-67 (MIB1) were purchased from LSBio (Seattle, WA, USA) and Dako (Carpinteria, CA, USA), respectively. The antigen–antibody complex was visualized with 3,3′-diaminobenzidine solution and counterstained with hematoxylin. Immunohistochemistry for ER (CONFIRM anti-ER [SP1]) and progesterone receptor (PR) (CONFIRM anti-PR [1E2]; Roche Diagnostics Japan, Tokyo, Japan) was performed with Ventana Benchmark XT (Roche Diagnostics Japan), and that for HER2 was performed by HercepTest (Dako).

Scoring of immunoreactivity and subgroup definition of the breast carcinoma. CITED2 immunoreactivity was detected in the nucleus of carcinoma cells and was evaluated using the H-scoring system with some modifications.[13] Briefly, the H-score was generated by adding together 2× the percentage of strongly stained nuclei, 1× the percentage of weakly stained nuclei and 0× the percentage of negative nuclei, giving a range of 0–200. The CITED2 H-score in the non-neoplastic glands was similarly evaluated as for the carcinoma cells.

For ER, PR and Ki-67 immunostaining, the percentage of immunoreactivity (labeling index [LI]) was determined. Cases with ER or PR LI of more than 1% were considered ER-positive or PR-positive breast carcinoma.[14] HER2 immunostaining was scored according to the standardized HercepTest scoring system, and the score 3+ was considered positive. Intrinsic subtype was defined according to the 2011 St Gallen surrogate definition[15] as follows: luminal A (ER and/or PR positive, HER2 negative, Ki-67 LI < 14%), luminal B (ER and/or PR positive, HER2 negative, Ki-67 LI ≥ 14% [HER2 negative], or ER and/or PR positive, HER2 positive [HER2 positive]), HER2 positive (ER and PR negative, HER2 positive), and triple negative (ER, PR, HER2 negative).

Cell lines. The human breast carcinoma cell line MCF-7 and SKBR-3 were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan) and the American Type Culture Collection (ATCC; Manassas, VA, USA), and these cells were cultured in RPMI-1640 (Wako, Osaka, Japan) and McCoy 5A (Gibco, Rockville, MD, USA) containing 10% FBS (Gibco), respectively. Both MCF-7 and SKBR-3 were retro-authenticated by ATCC with short tandem repeat profiling and confirmed to be the original cell line (in 2016).

Real-time PCR. Total RNA was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA), and cDNA was synthesized using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). Real-Time PCR was carried out using the THUNDERBIRD SYBR qPCR Mix (TOYOBO). The primer sequences of CITED2 and the ribosomal protein L13A (RPL13A) were: CITED2 (NM_006079), 5′-GGTTTGGACCGCATCAAG-3′ (forward) and 5′-GATCGAGTCAACAGCTCACTCT-3′ (reverse); and RPL13A (NM_012423), 5′-CCTGGAGGAGAAG AGGAAAGAGA-3′ (forward) and 5′-TTGAGGACCTC TGTGTATTTGTCAA-3′ (reverse). The CITED2 mRNA level was calculated as the ratio of the RPL13A mRNA level in this study.

Immunoblotting. Total protein was extracted using M-PER (Thermo Fisher Scientific Pierce Biotechnology, Rockford, IL, USA). The lysate proteins (15 μg) were subjected to SDS–PAGE (10% acrylamide gel) and transferred onto Hybond PVDF membranes (GE Healthcare, Buckinghamshire, UK). The primary anti-CITED2 antibody used was the same as that in the immunohistochemistry (LS-B243, LSBio), and anti-p53 antibody (DO-1) was purchase from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-β-actin antibody (A3854, Sigma-Aldrich) was also used as an internal control. In this study the p53 protein level was calculated as the ratio of the β-actin protein level.

Plasmid transfection. CITED2 expression plasmid was constructed by inserting a full-length open-reading frame of human CITED2 mRNA (NM_006079) into the vector pcDNA3.1 (−) (Invitrogen, Carlsbad, CA, USA) using the restriction enzymes Apa1 and Kpn1 (CITED2 plasmid). The plasmid was transfected into MCF-7 and SKBR-3 cells using Lipofectamin 3000 Reagent (Invitrogen). As a control, empty vector pcDNA3.1 (−) was transfected in this study.

siRNA transfection. Two siRNA oligonucleotides for CITED2 used in this study were designed as follows: siCITED2-1 (sense: 5′-UUAUGUCCUUGGUGAUAGATT-3′; antisense: 5′-UCUAUCACCAAGGACAUAATT-3′) and siCITED2-2 (sense: 5′-UGACGGACUUCGUGUGCAATT-3′; antisense: 5′-UUGCACACGAAGUCCGUCATT-3′). These were purchased from Sigma-Aldrich. MISSION siRNA Universal Negative Control (Sigma-Aldrich) was used as a negative control (siCTRL). The siRNA (10 nM) were transfected using the Lipofectamine 3000 Reagent (Invitrogen) according to the manufacturer's protocol.

Cell proliferation and wound healing assay. MCF-7 and SKBR-3 were transfected with CITED2 plasmid or CITED2-specific siRNA in 96-well culture plates. The cell proliferation status was measured by the WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) method using Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan) 0–4 days after the transfection in these cells.

The migration property of MCF-7 and SKBR-3 cells transfected with CITED2 plasmid or CITED2 siRNA was evaluated by wound scratch healing assay using Culture-Inserts (Ibidi GmbH, Munich, Germany). After cell adherence, the Culture-Inserts were removed and the remaining gaps were evaluated under light microscopy and quantified using NIS Elements software v3.0 (Nikon, Tokyo, Japan).

Chemoresistace assay. To evaluate fluctuation of chemoresistance according to the expression level of CITED2 in MCF-7 and SKBR-3 cells, the cells transfected with CITED2 plasmid or CITED2 siRNA were treated with 1 μM EPI (Wako),

10 μg/mL 5FU (Wako), a combination of EPI (1 μM) and 5FU (1 μg/mL) or 5 nM PTX (Wako). These are frequently used for breast cancer patients, and the concentrations were determined by referring to previous reports.[16–18] Three days after the incubation, relative cell viability was calculated as the ratio of treated cells to nontreatment cells the WST-8 assay.[19,20]

Statistical analysis. CITED2 status and clinicopathological factors were evaluated by a Mann–Whitney *U*-test or a cross-table using the χ^2-test. Disease-free and breast cancer-specific survival curves were generated according to the Kaplan–Meier method, and statistical significance was calculated using the log-rank test. Univariate and multivariate analyses were evaluated using a proportional hazard model (Cox). *P*-values < 0.05 and 0.05 ≤ *P*-values < 0.10 were considered significant and borderline significant in this study, respectively.[21] In *in vitro* experiments, statistical analyses were performed using Student's *t*-test and Fisher's protected least significant difference test.

Results

CITED2 immunolocalization in human breast carcinoma. CITED2 immunoreactivity was detected in the nucleus of breast carcinoma cells (Fig. 1a,b). CITED2 was also immunolocalized in the morphologically normal glands, but not in the stroma (Fig. 1c). The CITED2 H-score was significantly ($P = 0.0007$ by a Mann–Whitney *U*-test) higher in the breast carcinoma (the median with min-max value: 41 with range of 5–160) than the non-neoplastic mammary glands adjacent to the carcinoma (40 with range of 5–65) (Fig. 1d). The CITED2 H-score was also significantly higher in the breast carcinoma than the corresponding non-neoplastic mammary glands of the same case in the 80 paired samples examined ($P = 0.0005$ by a Wilcoxon signed rank test) (data not shown). Because almost all (90th percentile) non-neoplastic mammary glands revealed an H score ≦60 for CITED2, cases with an H score of more than 60 were considered CITED2-high breast carcinoma in this study.[22]

Associations between immunohistochemical CITED2 status and clinicopathological parameters in the breast carcinoma cases are summarized in Table 1. The CITED2 status was significantly associated with stage ($P = 0.025$), pathological T factor (pT) ($P = 0.045$), lymph node metastasis ($P = 0.017$), histological grade ($P = 0.021$), HER2 status ($P = 0.024$), Ki-67 LI ($P = 0.0082$) and intrinsic subtype ($P = 0.028$), while it was inversely correlated with ER status ($P = 0.033$). No significant association was detected between CITED2 status and other factors examined in this study.

Association between CITED2 status and clinical outcome of the patients. As shown in Figure 2(a), CITED2 status was significantly associated with an increased incidence of recurrence in breast cancer patients ($P < 0.0001$). A significant association was also detected between CITED2 status and an adverse clinical outcome of these patients ($P = 0.0089$) (Fig. 2b). A similar tendency was detected regardless of the sample collection period (1995–1999) in which the aromatase inhibitor had not yet been used (Fig. 2c,d). Significant associations between CITED2 status and disease-free survival were also detected in the cases with lymph node metastasis ($P = 0.048$) (Fig. 2e) and in pT2-4 cases ($P = 0.024$) (Fig. 2f). Moreover, CITED2 status was significantly associated with recurrence in the patients who received adjuvant chemotherapy ($P = 0.016$) (Fig. 2g) or endocrine therapy ($P = 0.0024$) (Fig. 2h).

The results of univariate analysis of disease-free survival using Cox (Table 2), Ki-67, pT, CITED2, PR, lymph node metastasis and histological grade were demonstrated to be significant prognostic factors for disease-free survival and ER was determined to be borderline significant. Multivariate analysis revealed that CITED2 ($P = 0.0036$), Ki-67 ($P = 0.0042$) and PR ($P = 0.029$) were independent prognostic factors.

As shown in Table 3, univariate analyses for breast cancer-specific survival revealed Ki-67, PR, histological grade, pT, lymph node metastasis and CITED2 as significant prognostic variables in these patients, and ER was determined to be borderline significant. Subsequent multivariate analysis revealed that PR ($P = 0.016$), Ki-67 ($P = 0.030$) and CITED2

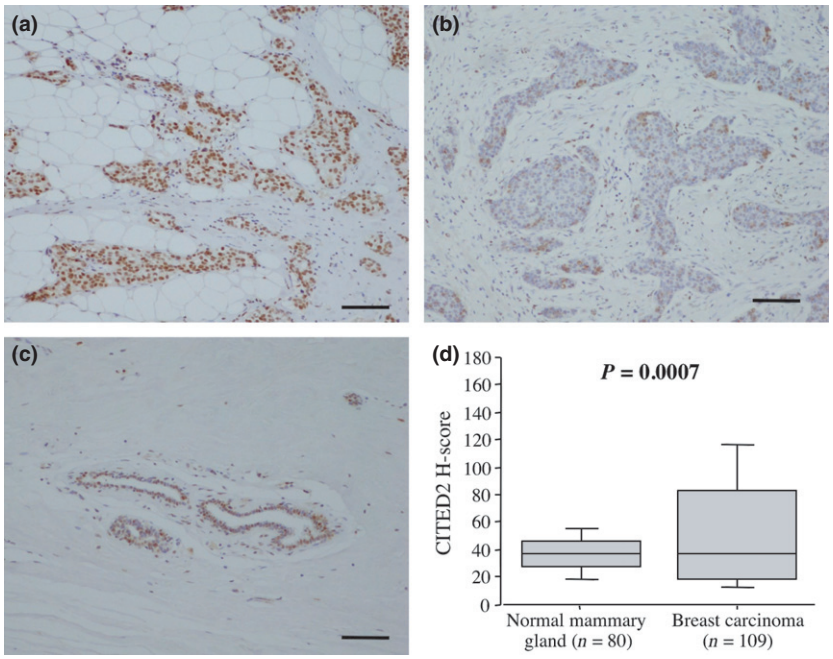


Fig. 1. Immunolocalization of CITED2 in breast carcinoma. CITED2 was immunolocalized in the nuclei of carcinoma cells. (a) CITED2-high case (CITED2 H-score = 140) and (b) CITED2-low cases (CITED2 H-score = 12). (c) CITED2 immoreactivity was weakly and focally detected in the epithelium of the morphologically normal mammary gland (CITED2 H-score = 35). Bar = 100 μm, respectively. (d) CITED2 H-score in breast carcinoma compared with that in non-neoplastic mammary glands. Data are represented as box and whisker plots. Median value is represented by a horizontal line in each box, and the 75th (upper margin) and 25th (lower margin) percentiles of the values are demonstrated. The upper and lower bars indicate the 90th and 10th percentiles, respectively.

Table 1. Association between CITED2 immunoreactivity and clinicopathological parameters in 109 breast carcinoma cases

	CITED2 status		*P*-value
	High (n = 38)	Low (n = 71)	
Age† (years)	56 (27–82)	56 (36–87)	0.28
Menopausal status			
Premenopausal	13	26	0.66
Postmenopausal	25	45	
Stage			
I	15	45	0.025*
II	12	18	
III	11	8	
Pathological T factor (pT)			
pT1	20	51	0.045*
pT2–4	18	20	
Lymph node metastasis			
Positive	19	18	0.017*
Negative	19	53	
Histological grade			
1 (well)	7	35	0.021*
2 (moderate)	17	26	
3 (poor)	14	10	
ER status			
Positive	26	61	0.033*
Negative	12	10	
PR status			
Positive	22	52	0.11
Negative	16	19	
HER2 status			
Positive	10	7	0.024*
Negative	28	64	
Ki-67 LI† (%)	17 (1–53)	9 (1–60)	0.0082*
Intrinsic subtype			
Luminal A	13	43	0.028*
Luminal B	12	18	
HER2	6	3	
Triple negative	7	7	

†Data represent the median (min–max), and the statistical analyses were performed using Mann–Whitney's *U*-test. All other values are presented as the number of cases, and the statistical analyses were performed using a cross-table applying the χ^2-test. *P-value < 0.05 was considered significant.

(P = 0.042) were independent parameters of the patients in this study.

Effects of CITED2 expression on cell proliferation and migration in breast carcinoma cells. To examine the biological functions of CITED2 in human breast carcinoma cells, we transfected CITED2 plasmid into ER-positive MCF-7 and ER-negative SKBR-3 cells. As shown in the upper panels of Figure 3(a), CITED2 mRNA expression levels were significantly increased in the cells transfected with CITED2 plasmid compared to those transfected with control plasmid ($P < 0.001$ in MCF-7 and $P < 0.001$ in SKBR-3). Accordingly, CITED2 protein levels were markedly increased in these cells transfected with CITED2 plasmid under the same conditions (Fig. 3a, lower panels).

The effects of CITED2 expression on cell proliferation in breast carcinoma cells are summarized in Figure 3(b). The number of cells was significantly increased in MCF-7 cells transfected with CITED2 plasmid from 3 to 4 days after the transfection (Day 3; $P < 0.05$ and Day 4; $P < 0.001$) compared to the control cells transfected with control plasmid. Similar tendency was also detected in SKBR-3 cells under the same conditions (Day 3; $P < 0.01$ and Day 4; $P < 0.05$). When we performed a wound healing assay in these cells, relative migration areas in MCF-7 and SKBR-3 cells transfected with CITED2 plasmid were significantly decreased compared to their controls (MCF-7; $P < 0.05$ at 24 h and $P < 0.01$ at 36 h, and SKBR-3; $P < 0.05$ at 96 h) (Fig. 3c). As shown in Figure S1, transfection of CITED2 plasmid did not significantly influence the estrogen-meditated proliferation (P = 0.74) and migration properties (P = 0.32) in MCF-7 cells in this study.

Because CITED2 expression was abundant in MCF-7 cells (Fig. 3a), we next transfected specific siRNA for CITED2 into MCF-7 cells. The mRNA expression levels of CITE2 were significantly decreased in MCF-7 cells transfected with specific CITED2 siRNA (siCITED2-1 or siCITED2-2) 3 days after transfection compared with those in cells transfected with negative control siRNA (siCTRL) (Fig. 3d, upper panel). Decreased protein levels of CITED2 were confirmed by immunoblotting under the same conditions (Fig. 3d, lower panel). MCF-7 cells transfected with CITED2 siRNA showed significant decrease in the cell proliferation activity from 3 to 4 days after the transfection (Fig. 3e) and migration area 36 h after removal of the culture insert (Fig. 3f).

Our immunohistochemical analysis showed a significant association between CITED2 and HER2 status in the breast carcinoma (Table 1). HER2 mRNA level was increased 2.0-fold in HER2-positive SKBR-3 cells transfected with CITED2 plasmid compared to the control cells, but it did not reach significance (P = 0.12) in this study (Fig. S2).[23]

Effects of CITED2 on chemoresistance in breast carcinoma cells. In our immunohistochemical study, CITED2 status was significantly associated with worse clinical outcome of breast cancer patients regardless of the adjuvant chemotherapy after the surgery (Fig. 2g). However, to the best of our knowledge, the effects of CITED2 on chemoresistance have not been reported in breast carcinoma. As shown in Figure 4(a), relative cell viability was significantly higher ($P < 0.001$ and $P < 0.05$) in MCF-7 and SKBR-3 cells transfected with CITED2 plasmid compared to the cells transfected with control plasmid under the treatment of EPI. A similar tendency was detected in these cells transfected with CITED2 plasmid under 5FU treatment (Fig. 4b) or combination treatment with EPI and 5FU (Fig. 4c). However, the relative cell viability was not significantly changed in these cells under the PTX treatment in this study (Fig. 4d).

As shown in Figure 4(e), the relative cell viability tended to be decreased in the MCF-7 cells transfected with CITED2 siRNA compared to the cells transfected with siCTRL under the treatment with EPI, 5FU and their combination, although

Fig. 2. Disease-free (a,c,e–h) and breast cancer-specific survival (b,d) of breast cancer patients according to CITED2 status. (a,b) Whole cases (n = 109). (c,d) Cases underwent surgery from 1995 to 1999 in Tohoku University Hospital (n = 56). (e) Cases positive for lymph node metastasis, (f) pT2-4 cases, (g) cases received adjuvant chemotherapy after surgery and (h) cases received adjuvant endocrine therapy following surgery. The solid line shows CITED2-high cases and the dashed line shows CITED2-low cases. *P*-values < 0.05 were considered significant and are shown in bold. *P*-value was not estimated (NE) in (d), because no patients had died in a CITED2-low group in this study.

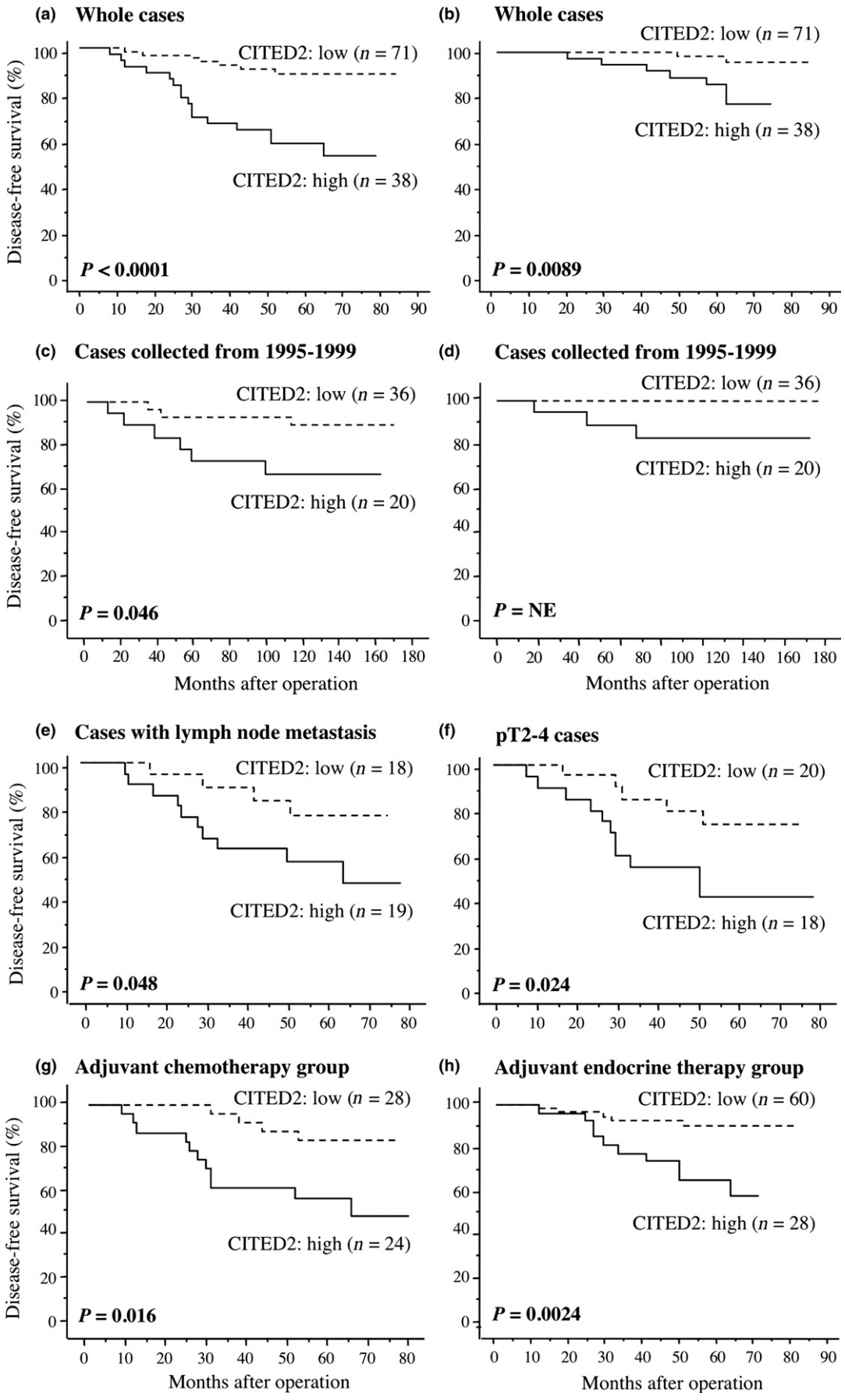


Table 2. Univariate and multivariate analyses of disease-free survival in 109 breast cancer patients examined

Variable	Univariate		Multivariate	
	P-value	Relative risk (95% CI)	*P*-value	Relative risk (95% CI)
Ki-67 LI† (0–60)	<0.0001**	1.05 (1.03–1.08)	0.0042	1.05 (1.02–1.09)
pT (pT1/pT2–4)	0.0003**	5.20 (2.13–12.70)	0.080	2.98 (0.84–10.63)
CITED2 status (Low/high)	0.0004**	4.93 (2.02–12.00)	0.0036	3.92 (1.56–9.83)
PR status (Negative/positive)	0.0021**	0.27 (0.12–0.62)	0.029	0.29 (0.10–0.88)
Lymph node metastasis (Negative/positive)	0.0040**	3.43 (1.48–7.94)	0.99	0.97 (0.32–3.04)
Histological grade (1,2/3)	0.0070**	3.11 (1.36–7.12)	0.11	2.84 (0.93–8.73)
ER status (Negative/positive)	0.050*	0.42 (0.18–1.00)	0.36	0.55 (0.18–1.70)
HER2 status (Negative/positive)	0.26	0.43 (0.10–1.83)		

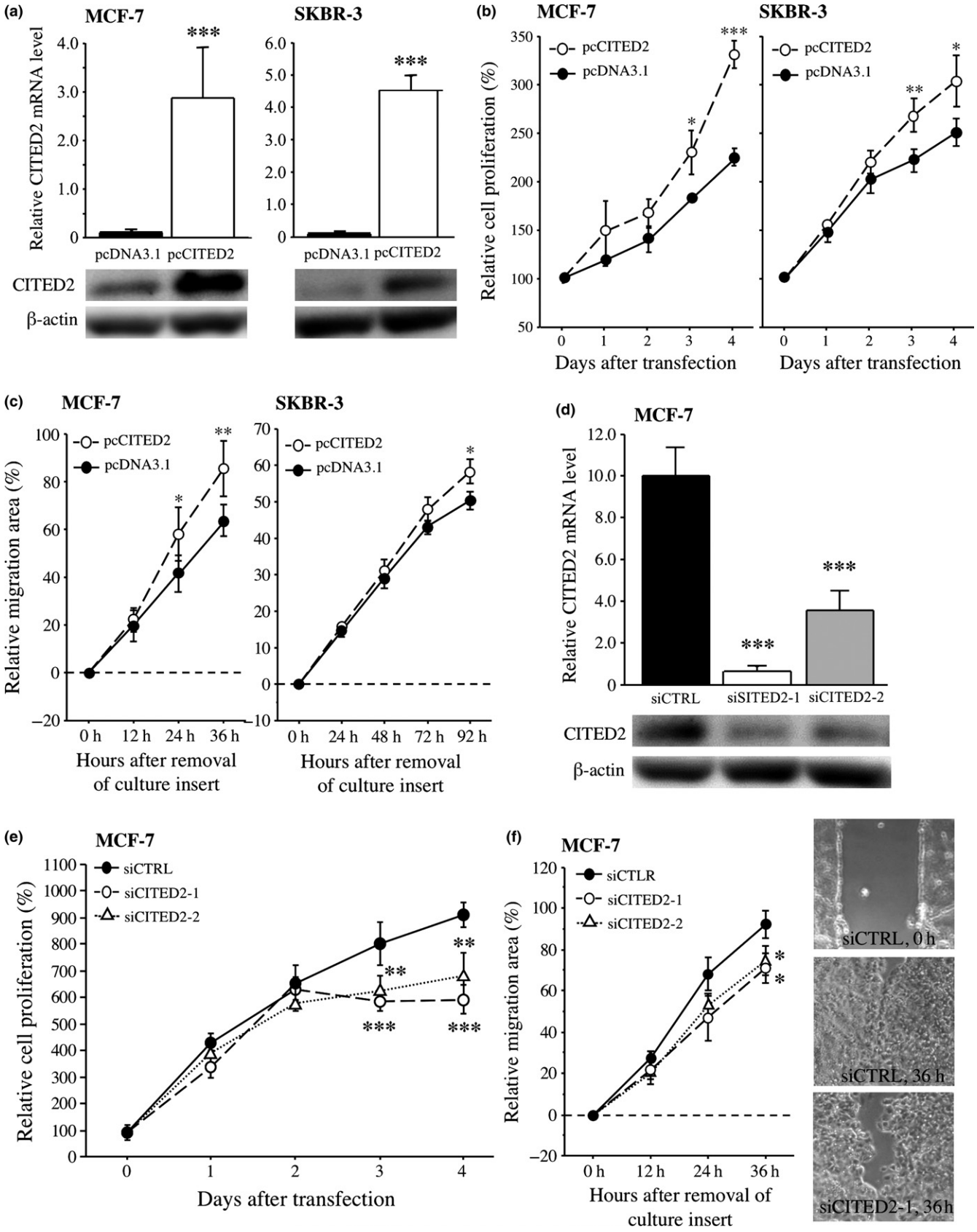
Statistical analysis was evaluated by a proportional hazard model (Cox). ***P* value < 0.05 and 0.05 ≤ **P*-value < 0.10 were considered significant and borderline significant respectively, and these values were examined in the multivariate analyses in this study. †Data were evaluated as continuous variables, and all other data were evaluated as dichotomized variables. 95% CI, 95% confidence interval.

Table 3. Univariate and multivariate analyses of breast cancer-specific survival in 109 breast cancer patients examined

Variable	Univariate		Multivariate	
	P-value	Relative risk (95% CI)	*P*-value	Relative risk (95% CI)
Ki-67 LI† (0–60)	0.0001**	1.07 (1.03–1.11)	0.030	1.07 (1.01–1.14)
PR status (Negative/positive)	0.0078**	0.60 (0.07–0.48)	0.016	0.050 (0.01–0.57)
Histological grade (1,2/3)	0.0081**	6.50 (1.63–25.99)	0.74	1.91 (0.33–11.00)
pT (pT1/pT2–4)	0.014**	7.22 (1.50–34.81)	0.68	2.21 (0.20–24.94)
Lymph node metastasis (Negative/positive)	0.017**	6.77 (1.41–32.61)	0.16	3.98 (0.58–27.19)
CITED2 status (Low/high)	0.023**	6.19 (1.28–29.80)	0.042	11.14 (1.09–113.98)
ER status (Negative/positive)	0.072*	0.30 (0.08–1.12)	0.25	0.38 (0.09–1.68)
HER2 status (Negative/positive)	0.60	0.57 (0.07–4.58)		

Statistical analysis was evaluated by a proportional hazard model (Cox). ***P* value < 0.05 and 0.05 ≤ **P*-value < 0.10 were considered significant and borderline significant respectively, and these values were examined in the multivariate analyses in this study. †Data were evaluated as continuous variables, and all other data were evaluated as dichotomized variables. 95% CI, 95% confidence interval.

Fig. 3. Effects of CITED2 on cell proliferation and migration properties in breast carcinoma cells. (a) Expression of CITED2 mRNA evaluated by real-time PCR in MCF-7 (left upper panel) and SKBR-3 (right upper panel) cells transfected with CITED2 plasmid (pcCITED2; open bar) or control plasmid (pcDNA3.1; closed bar). Lower panels show the corresponding CITED2 immunoreactivity in MCF-7 (left) and SKBR-3 (right) cells by immunoblotting. (b) Proliferation activity of MCF-7 (left) and SKBR-3 (right) cells transfected with CITED2 plasmid summarized as a ratio compared to that at 0 days after treatment. (c) Wound healing assays in MCF-7 (left) and SKBR-3 (right) cells. The relative migration area was evaluated as a ratio compared to that at 0 h after removal of culture insert. (d) Expression of CITED2 mRNA evaluated by real-time PCR in MCF7 cells (upper) transfected with CITED2-specific siRNA (siCITED2-1 [open bar] and siCITED2-2 [gray bar]) or negative control siRNA (siCTRL; closed bar). Lower panels show the corresponding CITED2 immunoreactivity in MCF-7 cells by immunoblotting. (e) Proliferation activity of MCF-7 cells transfected with siRNA summarized as a ratio compared to that at 0 days after treatment. (f) Wound healing assays in MCF-7 cells. The relative migration area was evaluated as a ratio compared to that at 0 h after removal of culture insert. Right panels show representative microphotographs under the indicated condition. In all figures, data were presented as the mean ± SD ($n = 3$), and statistical analyses were performed compared to the control cells transfected with control plasmid or control siRNA. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.



P-values did not reach significance in some groups. In contrast, PTX treatment did not significantly alter the relative cell viability in these cells (Fig. 4e). We could not examine the chemoresistance assay in SKBR-3 cells transfected with CITED2 siRNA, because the CITED2 mRNA level was negligible in SKBR-3 cells, as shown in Figure 3(a).

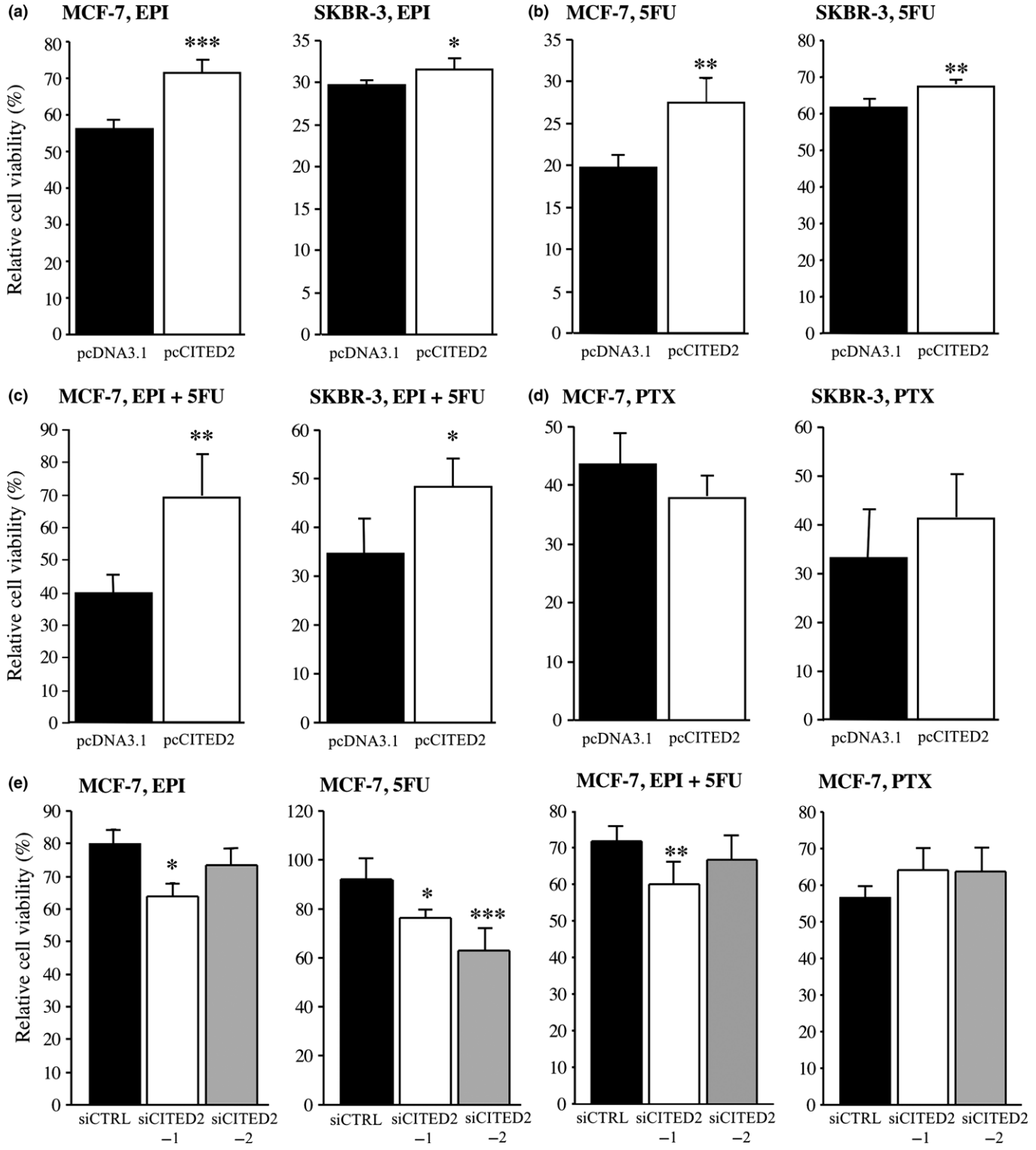


Fig. 4. Effects of chemotherapy on cell viability of breast carcinoma cells according to CITED2 expression level. (a–d) MCF-7 (left) and SKBR-3 (right) cells were transfected with CITED2 plasmid (open bars) or control plasmid (closed bars) and treated with epirubicin (EPI) (a), 5-fluorouracil [5FU] (b), combination of EPI and 5FU (c) and paclitaxel (PTX) (d) for 3 days. (e) MCF-7 cells transfected with siCITED2-1 (open bars), siCITED2-2 (gray bars) or siCTRL (closed bars) were treated with EPI, 5FU, combination of EPI and 5FU and PTX for 3 days. Relative cell viability was calculated as the ratio of that in the nontreatment cells using the WST-8 assay. In all figures, data are presented as the mean ± SD ($n = 3$). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

Inhibition of p53 accumulation by CITED2 in MCF-7 cells after 5-fluorouracil treatment. Our present *in vitro* results showed that CITED2 was involved in the resistance to EPI and 5FU (which are known to cause DNA damage)[24,25] in breast carcinoma cells (Fig. 4). Because DNA damage induces p53 protein accumulation and p53-dependent apoptosis,[26] we next examined

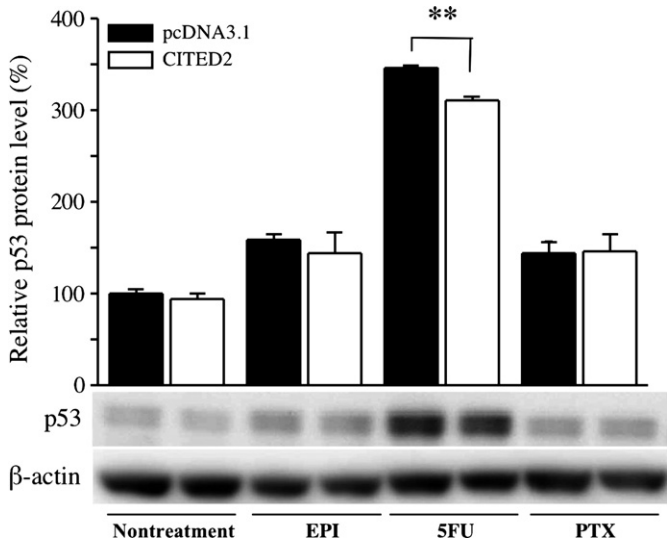


Fig. 5. Effects of chemotherapy on p53 protein level of MCF-7 cells according to CITED2 expression level. MCF-7 cells were transfected with CITED2 plasmid (open bars) or control plasmid (closed bars) and treated with epirubicin (EPI), 5-fluorouracil (5FU) or paclitaxel (PTX) for 3 days. Subsequently, immunoblotting for p53 was performed. Upper panel showed relative p53 protein level evaluated as a ratio compared with the control cells (i.e. nontreatment MCF-7 cells transfected with control plasmid; left bar). Data were presented as the mean ± SD ($n = 3$). $^{**}P < 0.01$. Lower panel shows immunoblotting for p53.

the possible regulation of p53 expression by CITED2 under chemotherapy in MCF-7 cells with wild-type p53.[27]

As shown in Figure 5, the p53 protein level in MCF-7 cells transfected with CITED2 plasmid was similar to that in the control MCF-7 cells (97%) when these cells were not treated with the chemotherapy drug. The p53 protein level was accumulated after EPI and 5FU treatment for 3 days, but it was significantly lower ($P < 0.01$) after 5FU treatment. A similar tendency was detected after EPI treatment, although it did not reach significance ($P = 0.19$). In contrast, the p53 protein level was similar in MCF-7 cells transfected with CITED2 plasmid compared to the cells transfected with control plasmid after PTX treatment (111%).

Discussion

This is the first study to demonstrate the clinical significance of CITED2 immunoreactivity in breast carcinoma. In this study, CITED2 immunoreactivity was significantly higher in breast carcinoma in comparison with non-neoplastic mammary glands. Previously, Lau *et al.*[11] show that expression of CITED 2 mRNA was significantly elevated in invasive ductal carcinoma samples relative to normal mammary epithelium, which is in good agreement with our present results. CITED2 is a downstream target of MYC oncogene[28] and it is also induced by RAS oncogene through tumor susceptibility gene 101 (TSG101).[29] In addition, Sun *et al.*[30] show that overexpressed CITED2 in Rat1 fibroblasts caused tumor formation with fibrosarcoma-like characteristics in nude mice. Therefore, it is suggested that CITED2 protein is increased in the process of the mammary carcinogenesis and plays important roles in breast carcinomas.

In the present study, CITED2 immunoreactivity was significantly associated with Ki-67 LI and histological grade in the breast carcinoma. Ki-67 LI reflects the proliferative activity of breast carcinoma,[31] while histological grade is evaluated by the mitotic rate, nuclear atypia and tubule formation of breast carcinoma. Moreover, the results of *in vitro* experiments demonstrated that CITED2 expression level was significantly associated with the proliferation activity of MCF-7 and SKBR-3 cells. Sun *et al.*[30] report increased anchorage-independent growth by overexpressed CITED2 in Rat1 fibroblasts, and Chou *et al.*[28] show that CITED2 significantly promoted the growth of lung carcinoma xegnografts through CITED2/MYC/E2F3/p21 pathway. The present results are consistent with these reports, and it is suggested that CITED2 plays an important role in the cell proliferation of breast carcinoma.

The present study also revealed that CITED2 status was significantly associated with pT in breast carcinoma and CITED2 expression level was significantly associated with the migration property in MCF-7 and SKBR-3 cells. Chou *et al.*[32] report that knockdown of CITED2 in MDA-MB-231 breast carcinoma cells attenuated transforming growth factor β1 (TGFβ1)-mediated upregulation of matrix metalloproteinase-9 (MMP9) and cell invasiveness *in vitro*, and Lau *et al.*[11] show that CITED2 caused osteolytic bone metastasis of breast carcinoma in animal models, possibly through its regulation of TGFβ1 action. CITED2-mediated invasiveness has been also reported in colon cancer cells.[33] Taking these previous results together with our present results, CITED2 appears to promote the invasion of breast carcinoma.

In the present study, CITED2 status was significantly associated with recurrence and worse prognosis in breast cancer patients, and the results of multivariate analyses demonstrated that CITED2 status was an independent prognostic factor for both disease-free and breast cancer-specific survival. Chou *et al.*[28] demonstrate that CITED2 knockdown increased overall host mouse survival rates in a lung carcinoma xenograft model, and CITED2 expression was significantly associated with poor prognosis of lung cancer patients, which is consistent with our results. The association between CITED2 and the clinical outcome of ER-positive breast cancer patients is currently controversial. van Agthoven *et al.*[10] show that CITED2 mRNA level was significantly associated with prolonged metastasis-free survival of lymph node-negative patients with ER-positive breast cancer. However, we previously found CITED2 to be linked to the recurrence after tamoxifen therapy in ER-positive breast cancer patients based on microarray data,[3] and Lau *et al.*[34] report that CITED2 mRNA expression in ER-positive breast carcinoma was higher in tumors from patients surviving less than 5 years from the time of diagnosis than those surviving >5 years. Lau *et al.*[34] also demonstrate that increased CITED2 expression resulted in estrogen-independent ER activation and reduced response to anti-estrogen therapy in breast carcinoma cell lines. The present results are consistent with later findings, and CITED2 may be, at least in a part, involved in the resistance to endocrine therapy of ER-positive breast carcinoma.

The association between CITED2 and chemoresistance has not been examined in breast carcinoma. Previous studies have shown that CITED2 modulates the effect of chemotherapy on some carcinoma cells, but these results are not necessarily consistent. For instance, CITED2 was overexpressed in KFR oxaliplatin-resistant ovarian carcinoma cells,[35] cisplatin-resistant HeLa cells[36] and LS174T irinotecan-resistant colorectal carcinoma cells.[37] In contrast, Ju *et al.*[38] report that CITED2 expression was downregulated in chemoresistant ovarian carcinomas and Regel *et al.*[39] show that gastric carcinoma cell

lines with a low CITED2 expression were more drug resistant to anthracyclines compared with those with high CITED2 expression. They also show that histone deacetylase (HDAC) inhibitor can overcome the resistance of mouse gastric carcinoma cells to anthracyclone by inducing expression of CITED2. These divergent findings may be partly due to the different types of carcinoma cells examined. In the present study, CITED2 status was associated with worse prognosis of patients who received adjuvant chemotherapy, and *in vitro* studies demonstrated that CITED2 caused EPI and 5FU-resistant proliferation of MCF-7 and SKBR-3 cells. Therefore, CITED2 is suggested to play an important role in the chemoresistance of breast carcinoma.

Moreover, our *in vitro* studies showed that CITED2 inhibited p53 accumulation after 5FU treatment in MCF-7 cells with wild type p53. EPI and 5FU are known to cause DNA damage,[24,25] and it induces accumulation of p53 tumor suppressor protein and p53-dependent apoptosis.[26,40] Previously, Wu *et al.*[36] report that knockdown of CITED2 sensitized cancer cells to cisplatin through stabilization of p53 and enhancement of p53-dependent apoptosis, which is consistent with our findings. However, CITED2 was not associated with the resistance to PTX in the breast carcinoma cells in our study. PTX binds to tubulin and inhibits the disassembly of microtubules, and PTX-induced apoptosis is independent of p53 activity.[41,42] Therefore, it is suggested that CITED2 causes chemoresistance of breast carcinoma through inhibition of p53-dependent apoptosis in the breast carcinoma. However, CITED2 was associated with resistance to EPI and 5FU also in SKBR-3 cells with mutant p53[43] in our study, and recent studies have demonstrated that CITED2 is involved in the maintenance of stem cells.[44–46] Because CITED2 expression is associated with an aggressive phenotype of breast carcinoma through regulating a variety of biological functions, as described in this section, residual carcinoma cells following surgical treatment in CITED2-positive breast carcinomas could still have the potential to rapidly recur despite the adjuvant therapies. Further examinations are required to clarify the molecular mechanism of CITED2 associated with resistance to the adjuvant therapy in breast cancer patients.

In summary, CITED2 immunoreactivity was significantly increased in breast carcinoma tissues, and it turned out to be an independent worse prognostic factor for the breast cancer patients. Subsequent *in vitro* experiments demonstrated that CITED2 significantly promoted the proliferation activity and migration property in MCF-7 and SKBR-3 cells. Moreover, CITED2 increased chemoresistance to EPI and 5FU in these cells, and inhibited p53 accumulation after 5FU treatment in MCF-7 cells. These results suggest that CITED2 plays important roles in the progression and chemoresistance of breast carcinoma and that CITED2 status is a potent prognostic factor in breast cancer patients.

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Disclosure Statement

The authors have no conflict of interest to declare.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Estrogen-mediated cell proliferation and migration activity in MCF-7 cells transfected with CITED2 plasmid.

Fig. S2. Expression of HER2 mRNA in SKBR-3 cells transfected with CITED2 plasmid.

Table S1. Clinicopathological characteristics of 109 breast carcinomas in the present study.