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From Cell Biology to Tissue Engineering

Cytochrome c1 as a favorable prognostic marker in estrogen receptor-positive breast carcinoma

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Summary. Background. Cytochrome c1 (CYC1) is a heme-containing subunit of mitochondria complex III and is mainly involved in cellular energy production. A recent study has demonstrated that CYC1 was overexpressed in breast carcinoma tissues and induced proliferation, migration and invasion of estrogen receptor (ER)-negative breast carcinoma cells. However, the clinical significance of CYC1 protein remains largely unclear in invasive breast carcinoma, and biological functions of CYC1 have not been reported in ER-positive breast carcinoma cells.

Materials and methods. We immunolocalized CYC1 in 172 invasive breast carcinomas and evaluated its clinical significance according to the ER-status. Subsequently, we examined the effects of CYC1 on proliferation, glycolysis and chemosensitivity to paclitaxel, which is one of the most common chemotherapeutic agents in breast cancer, in ER-positive breast carcinoma cells (MCF7 and T47D).

Results. CYC1 immunoreactivity was detected in 47% of ER-positive cases and 30% of ER-negative cases. Immunohistochemical CYC1 status was inversely associated with Ki67 in ER-positive cases, and it was a significantly favorable prognostic factor for both disease-free and breast cancer-specific survival of the patients. On the other hand, no significant association was detected between CYC1 status and clinicopathological factors in ER-negative cases. In *in vitro* experiments, MCF7 and T47D cells transfected specific siRNA for *CYC1* significantly increased cell proliferation activity, L-lactate production and cell viability after paclitaxel treatment.

Conclusion. These results suggest that CYC1 inhibits cell proliferation, glycolytic activity and increases chemosensitivity to paclitaxel in ER-positive breast carcinoma cells and that CYC1 status is a potent favorable prognostic factor in ER-positive breast cancer patients.

Key words: Breast Neoplasms, Chemosensitivity, Cytochrome c1, Estrogen Receptor, Prognosis

Introduction

Breast cancer is the most common malignant neoplasm in women worldwide. Estrogens play an important role in the progression of breast cancer, and a great majority (for instance, 84% by Brouckaert et al. (2012)) of breast carcinoma express estrogen receptor

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Abbreviations. CTL, control; Cox, proportional hazard model; CYC1, cytochrome c1; DAB, 3;3'-diaminobenzidine; DCIS, ductal carcinoma *in situ* of the breast; LI, labeling index; OXPHOS, oxidative phosphorylation; PR, progesterone receptor; pT, pathological T factor; RPL13A, ribosomal protein L13A; VDAC1, voltage-dependent anion channel 1; WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt

(ER). Invasive breast carcinoma is generally considered a systemic disease (Fisher and Anderson, 2010), and adjuvant therapies such as endocrine therapy and chemotherapy are frequently used after the surgical treatment. However, recurrence rate was approximately 10% after 5-year endocrine therapy in ER-positive early breast cancer (Djalalov et al., 2015), and about 25% of breast cancer patients who received adjuvant chemotherapy developed recurrence (Tevaarwerk et al., 2013). Therefore, it is very important to evaluate clinical and/or biological markers to predict recurrence after surgery in ER-positive breast cancer patients.

Cytochrome c1 (CYC1) is a heme-containing subunit of the mitochondria complex III (Duncan et al., 1994), and it is involved in the cellular ATP production through the oxidative phosphorylation (OXPHOS) pathway (Yang et al., 2016). Overexpression of *CYC1* mRNA has been previously demonstrated in human nasopharyngeal carcinoma (Fang et al., 2008) and osteosarcoma (Li et al., 2014). Recently, Chishiki et al. (2017) demonstrated that CYC1 was immunolocalized in 40% of the ductal carcinoma *in situ* of the breast (DCIS), and it regulates proliferation and apoptosis in ER-negative DCIS cells. In addition, Han et al. (2016) reported that CYC1 upregulated proliferation, migration and invasion properties of ER-negative breast carcinoma cells. These results suggest the importance of CYC1 in the progression of ER-negative breast carcinoma. Although Han et al. (2016) also immunolocalized CYC1 in breast carcinoma tissues, the sample number was very limited (n=26) and the clinical significance of the CYC1 protein remains largely unclear in invasive breast carcinoma. Moreover, the biological functions of CYC1 have not been reported in ER-positive breast carcinoma cells. Therefore, we performed immunohistochemistry for CYC1 in 172 invasive breast carcinoma tissues according to the ER status, and *in vitro* studies were subsequently performed using ER-positive breast carcinoma cells to explore clinical and biological significance of CYC1 in ER-positive invasive breast carcinoma.

Materials and methods

Patients and tissues

172 specimens of invasive ductal carcinoma, not other specified, of the breast (ER-positive; n=139, and ER-negative n=33) were obtained from Japanese female patients (age range; 27-87 years) who underwent surgical treatment from 2006 to 2008 in Tohoku University Hospital (Sendai, Japan). All the specimens had been fixed in 10% formalin and embedded in paraffin wax. The 91 patients received adjuvant chemotherapy, while the 139 patients (all of ER-positive breast cancer patients) received adjuvant endocrine therapy after surgery. The clinical outcome was evaluated by disease-free and breast cancer-specific survival of the patients and the mean follow-up time was 64 months (range; 3-108 months) in this study. Breast cancer-specific survival was defined as the time from surgery to death from breast cancer.

The research protocol was approved by the Ethics Committee at Tohoku University School of Medicine (approval number 2015-1-162).

Immunohistochemistry

Rabbit polyclonal antibody for CYC1 (PA5-25257) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Mouse monoclonal antibody for Ki67 (MIB1) and androgen receptor (AR) (AR441) were purchased from DAKO (Carpinteria, CA, USA). A Histofine kit (Nichirei Bioscience, Tokyo, Japan) was used for the immunohistochemistry. The antigen-antibody complex was visualized with 3,3'-diaminobenzidine (DAB) solution and counterstained with hematoxylin. We used human tissue of the stomach as a positive control (Chishiki et al., 2017) and normal rabbit IgG instead of the primary antibody as a negative control for CYC1 immunostaining in this study. Immunohistochemistry for ER (CONFIRM anti-ER (SP1), Roche Diagnostics Japan, Tokyo, Japan) and progesterone receptor (PR) (CONFIRM anti-PR (1E2), Roche Diagnostics Japan) was performed with Ventana Benchmark XT (Roche Diagnostics Japan), and that for HER2 was performed by HercepTest (DAKO).

Immunohistochemical CYC1 status was considered positive when immunolocalized in the cytoplasm of carcinoma cells, and the cases that had more than 10% of positive carcinoma cells (Chisiki et al., 2017). Cases with more than 1% positive cells were considered ER-positive or PR-positive breast carcinoma (Hammond et al., 2010). Immunoreactivity of Ki67 and AR were detected in the nuclei of carcinoma cells, and percentage of positive cells (labeling index; LI) was determined by counting more than 1000 cells. HER2 immunoreactivity was evaluated according to the grading system proposed in HercepTest (DAKO).

Cell line

Human ER-positive breast carcinoma cell lines MCF7 and T47D were obtained from Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan) and American Type Culture Collection (ATCC; Manassas, VA, USA), respectively. In addition, ER-negative MCF10DCIS.com was purchased from Asterand (Detroit, MI, USA). This cell line is a clonal breast cancer cell line derived from a xenograft originating from premalignant MCF10AT cells which forms comedo DCIS (Miller et al., 2000) and we used this cell line as a model of DCIS. These cells were cultured in RPMI-1640 (Sigma-Aldrich) containing 10% fetal bovine serum (FBS; Gibco).

Small interfering RNA (siRNA) transfection

Two siRNA oligonucleotides for siCYC1 were used

in this study according to a previous report (Chisiki et al., 2017), which were designed as follows: siCYC1-1 (5'-rGrCUrGUUrCrGrArCUrAUUUrCrCrCrATT-3') and siCYC1-2 (5'-rGUrCrArCUrGrCrGrGrGrArArGrGUrCUrCTT-3'). MISSION siRNA Universal Negative Control (Sigma-Aldrich, St. Louis, MO, USA) was used as a negative control (siCTL). The siRNAs were transfected using the Lipofectamine 3000 Reagent (Invitrogen).

Real-time PCR

Total RNA was extracted using TRI Regent (Molecular Research Center, Cincinnati, OH, USA), and cDNA was synthesized using a 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 *CYC1* and ribosomal protein L13A (*RPL13A*) were: *CYC1* (NM_001916), 5'-GAGGTGGAGGTTCAAGACGG-3' (forward) and 5'-TAGCTCGCACGATGTAGCTG-3' (reverse); and *RPL13A* (NM_012423), 5'-CCTGGAGGAGAAGAGGAAAGAGA-3' (forward) and 5'-TTGAGGACCTCTGTGTATTTGTCAA-3' (reverse). The *CYC1* mRNA level was calculated as the ratio of the *RPL13A* mRNA level in this study.

Immunoblotting

The mitochondrial protein was extracted from breast carcinoma cells according to a previous report (Clayton and Shadel, 2014). The lysate proteins (2 μg) were subjected to SDS-PAGE (10% acrylamide gel) and transferred onto Hybond PVDF membranes (GE Healthcare, Buckinghamshire, UK). Primary anti-CYC1 antibody used was the same as that in the immunohistochemistry. Anti-mitochondrial voltage-dependent anion channel 1 (VDAC1) antibody (sc-390996; Santa Cruz Biotechnology, Dallas, TX, USA) was used as an internal control for mitochondrial protein (Zhu et al., 2012). Antibody-protein complexes on the membrane were detected using ECL-prime Western blotting detection reagents (GE Healthcare) and visualized by a LAS-4000 image analyzer (Fuji Photo Film Co., Tokyo, Japan).

Cell proliferation, glycolysis and chemosensitivity assay

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.

Extracellular L-lactate, the end product of glycolysis, was detected using Glycolysis Cell-Based assay kit (Cayman Chemical, Ann Arbor, MI, USA). Two days after the siCYC1 transfection, culture medium was changed to serum free culture medium and incubated for 1 day. The L-lactate concentration was measured following the manufacturer's instructions and normalized by cell numbers.

To evaluate fluctuation of chemosensitivity to paclitaxel, according to *CYC1* expression in MCF7 andT47D cells, the cells transfected with *CYC1* siRNA were treated with paclitaxel (Wako, Osaka, Japan) (3 nM for 2 days in MCF7 and 0.3 nM for 3 days in T47D). The concentrations and treatment periods were referred to previous reports (Zoli et al., 2005, Minemura et al., 2016). After the treatment, relative cell viability was calculated as the ratio of that in the nontreatment cells using the WST-8 assay (Minemura et al., 2016).

Statistical analysis

Immunohistochemical CYC1 status and clinicopathological factors were evaluated by the Student's t-test or a cross-table using the chi-square 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. Uni- and multivariate analyses were evaluated

Table 1. Association between immunohistochemical CYC1 status and clinicopathological factors in 139 ER-positive breast carcinomas.

	CYC1 status		P value
	+(n=66)	-(n=73)	
Age† (years)	55.4±1.6	54.7±1.5	0.76
Menopausal status			
Premenopausal	26	33	
Postmenopausal	40	40	0.49
Stage			
I	37	40	
II+III	26	36	0.47
Pathological T factor (pT)			
pT1	47	45	
pT2-4	19	28	0.23
Lymph node metastasis			
Positive	22	21	
Negative	44	52	0.58
Histological grade			
1-2	60	61	
3	6	12	0.20
PR status			
Positive	55	59	
Negative	11	14	0.70
HER2 status			
Positive	4	9	
Negative	62	64	0.21
Ki67 LI† (%)	10.3±1.0	14.5±1.4	0.018*

†: Data are presented as mean ± SE. All other values represent the number of cases. *: denote statistically significant (P value <0.05). CYC1, cytochrome c1; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; LI, labeling index; PR, progesterone receptor.

by a proportional hazard model (Cox). P values<0.05 and 0.05≤P values<0.10 were considered significant and borderline-significant respectively (Sato-Tadano et al., 2013). In *in vitro* experiments, statistical analyses were performed using Student's t-test and Fisher's PLSD test. The statistical analyses were performed using the StatView 5.0J software (SAS Institute, Cary, NC, USA).

Results

CYC1 immunolocalization in invasive breast carcinoma according to ER status

Immunoreactivity of CYC1 was detected in the cytoplasm of breast carcinoma cells (Fig. 1A,B). It was also weakly present in non-neoplastic mammary glands, while it was almost negligible in stroma (Fig. 1C). As shown in Table 1, immunohistochemical CYC1 status was positive in 66 out of 139 ER-positive invasive breast carcinomas (47%), and it was inversely associated with Ki67 LI (P=0.018). However, no significant association was detected between CYC1 status and patient age, menopausal status, stage, pathological T factor (pT), lymph node metastasis, histological grade, PR status and HER2 status in this study.

We also immunolocalized CYC1 in 33 ER-negative invasive breast carcinomas. CYC1 was immunolocalized

Table 2. Association between immunohistochemical CYC1 status and clinicopathological factors in 33 ER-negative breast carcinomas.

	CYC1 status		P value
	+(n=10)	-(n=23)	
Age† (years)	62.5±3.1	61.1±2.7	0.77
Menopausal status			
Premenopausal	1	6	
Postmenopausal	9	17	0.30
Stage			
I	5	14	
II+III	5	9	0.56
Pathological T factor (pT)			
pT1	6	14	
pT2-4	4	9	0.96
Lymph node metastasis			
Positive	5	6	
Negative	5	17	0.18
Histological grade			
1-2	3	12	
3	7	11	0.24
HER2 status			
Positive	4	10	
Negative	6	13	0.85
Ki67 LI† (%)	20.2±3.8	28.2±3.6	0.20

†: Data are presented as mean ± SE. All other values represent the number of cases. Abbreviation: CYC1, cytochrome c1; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; LI, labeling index.

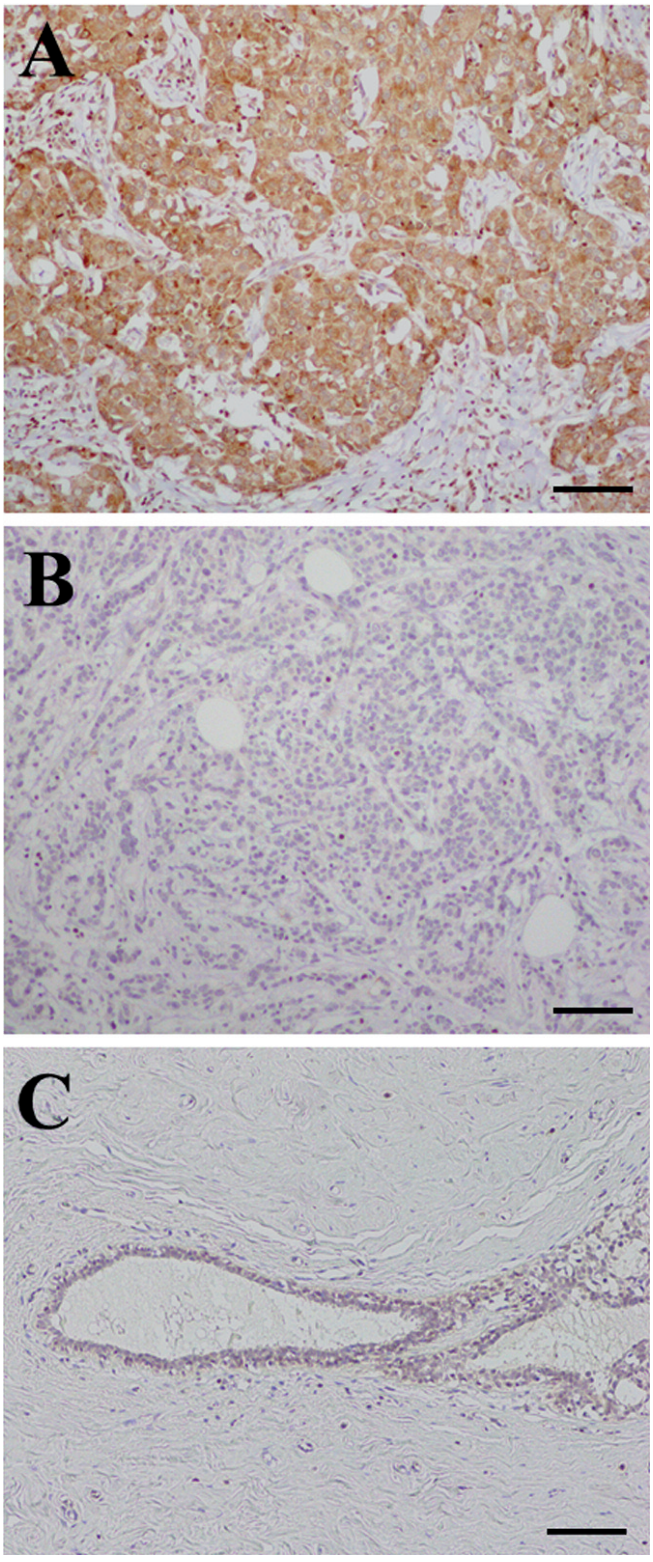

Fig. 1. Immunohistochemistry for CYC1 in invasive breast carcinoma. **A.** CYC1 was immunolocalized in the cytoplasm of breast carcinoma cells. **B.** CYC1-negative case. **C.** CYC1 immoreactivity was weakly observed in the morphologically normal mammary grand. Scale bars: 100 μm.

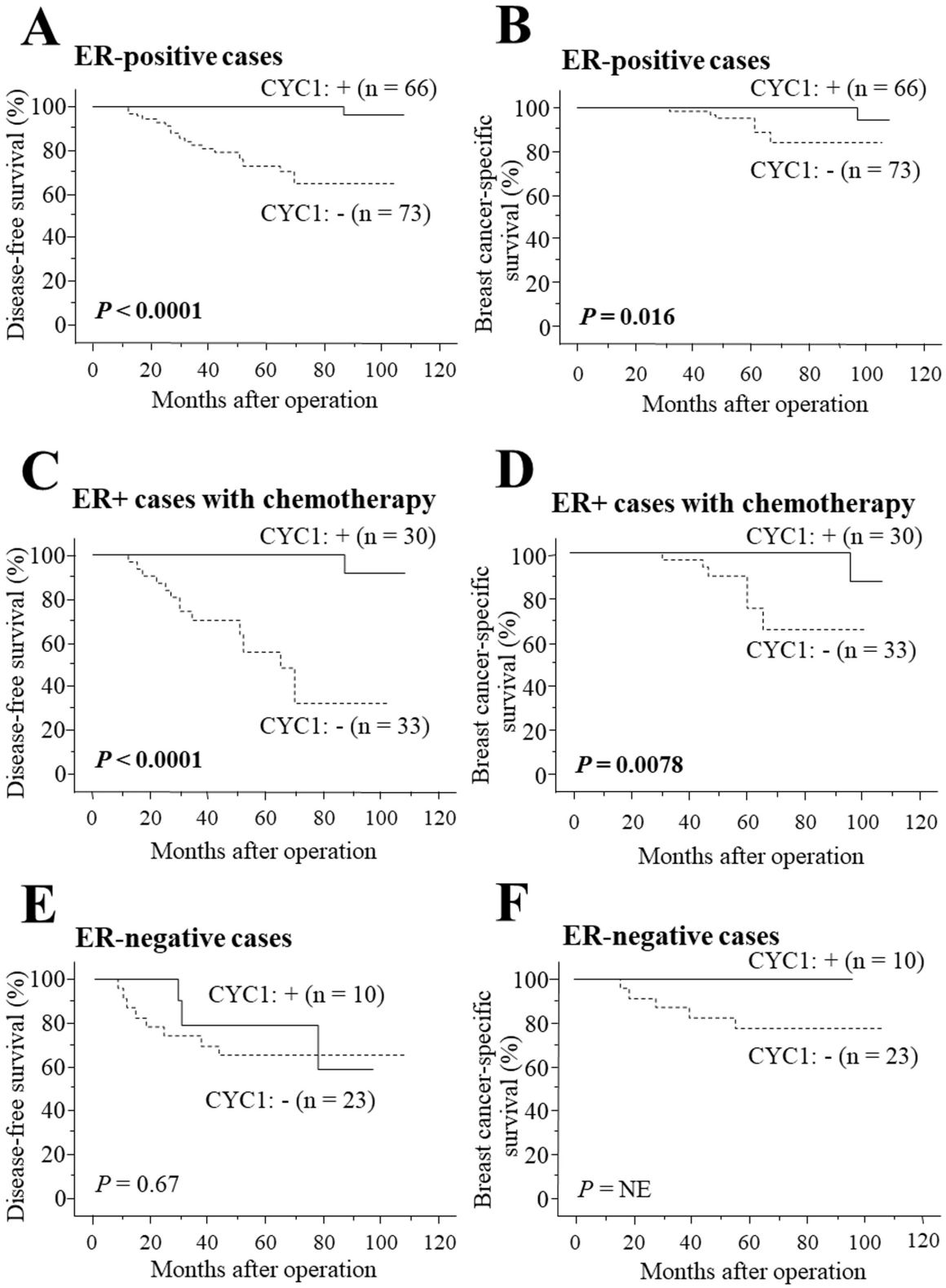

Fig. 2. Disease-free (**A, C, E**) and breast cancer-specific survival (**B, D, F**) of breast cancer patients according to CYC1 status. **A, B.** ER-positive cases (n=139). **C, D.** ER-positive breast cancer patients who received adjuvant chemotherapy (n=63). **E, F.** ER-negative cases (n=33). The solid line shows CYC1-positive group and the dashed line shows CYC1-negative group. P-values <0.05 were considered significant and shown in bold. P-value was not estimated (NE) in Figure 2F, because no patients had died in a CYC1-positive group.

in 10 out of the 33 (30%) ER-negative cases, but no significant association was detected between CYC1 status and various clinicopathological factors examined (Table 2). CYC1 status was marginally associated with ER status (P=0.070) in invasive breast carcinoma cases in total (n=172).

Association between CYC1 status and clinical outcome of breast cancer patients according to ER status

As shown in Fig. 2A,B, immunohistochemical CYC1 status was significantly associated with a decreased incidence of recurrence (P<0.0001 by log-rank test; Fig. 2A) and better breast cancer-specific survival after endocrine therapy (P=0.016 by log-rank test; Fig. 2B) of ER-positive breast cancer patients (n=139). A similar tendency was also detected in ER-positive breast cancer patients who received adjuvant chemotherapy (Fig. 2C,D). Results of univariate analysis of disease-free survival using Cox (Table 3), Ki67, pT, CYC1, histological grade and lymph node metastasis were demonstrated to be significant prognostic factors. Multivariate analysis revealed that CYC1 (P=0.0029) and Ki67 (P=0.049) turned out as independent positive and negative prognostic factors for disease-free survival respectively. As shown in Table 4, univariate analysis for breast cancer-specific survival revealed Ki67, histological grade, lymph node metastasis, pT and CYC1 as significant prognostic variables. Subsequent multivariate analysis demonstrated that lymph node metastasis (P=0.053) and CYC1 (P=0.095) were borderline significant factors, although no significant independent factors were detected in this study.

On the other hand, no significant association was detected between CYC1 status and clinical outcome of ER-negative breast cancer patients examined in this study (Fig. 2E,F).

Effects of CYC1 on proliferation, glycolysis and chemosensitivity in ER-positive breast carcinoma cells

In this study, CYC1 immunoreactivity was inversely associated with Ki67 LI and it turned out a positive prognostic factor in ER-positive breast carcinomas. However, biological functions of CYC1 have not been examined in ER-positive breast carcinoma cells to the best of our knowledge. Therefore, we next transfected specific siRNA for *CYC1* in MCF7 and T47D ER-positive breast carcinoma cells. The mRNA expression level of *CYC1* was significantly decreased in MCF7 cells transfected with siCYC1-1 (upper panel of Fig. 3A) and siCYC1-2 (upper panel of Fig. 3B) at 2 days after transfection. The protein level of CYC1 was also markedly decreased in relation to the mitochondrial protein VDAC1 in MCF7 cells transfected with siCYC1-1 (lower panel of Fig. 3A) and siCYC1-2 (lower panel of

Table 3. Univariate and multivariate analyses of disease-free survival in 139 ER-positive breast cancer patients.

	Univariate	Multivariate	
Variable	P value	P value	Relative risk (95% CI)
Ki67 LI (%)† (0-60)	<0.0001*	0.049*	1.04 (1.00-1.08)
pT (pT1/pT2-4)	0.0011*	0.11	2.27 (0.82-6.30)
CYC1 status (negative/positive)	0.0020*	0.0029*	0.04 (0.01-0.34)
Histological grade (1,2/3)	0.0029*	0.65	0.72 (0.18-2.88)
Lymph node metastasis (negative/positive)	0.0212*	0.082**	2.38 (0.9-6.3)
HER2 status (negative/positive)	0.96		
AR LI (%)† (0-94)	0.65		

Statistical analysis was evaluated by a proportional hazard model (Cox). * and ** denote statistically significant (P value<0.05) and borderline significant (0.05≤P value<0.10) respectively. Variables with P value of less than 0.05 in univariate analysis were further examined in multivariate analysis. †: Data were evaluated as continuous variables, and all other data were evaluated as dichotomized variables. AR, androgen receptor; CYC1, cytochrome c1; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; LI, labeling index; pT, pathological T factor; 95% CI, 95% confidence interval.

Table 4. Univariate and multivariate analyses of breast cancer-specific survival in 139 ER-positive breast cancer patients.

	Univariate	Multivariate	
Variable	P value	P value	Relative risk (95% CI)
Ki-67 LI (%)† (0-60)	<0.0001*	0.20	1.03 (0.98-1.09)
Histological grade (1,2/3)	0.0008*	0.44	2.20 (0.30-16.44)
Lymph node metastasis (negative/positive)	0.017*	0.053**	6.74 (0.97-46.66)
pT (pT1/pT2-4)	0.029*	0.59	1.62 (0.29-9.11)
CYC1 status (negative/positive)	0.044*	0.095**	0.12 (0.01-1.47)
HER2 status (negative/positive)	0.78		
AR LI (%)† (0-94)	0.46		

Statistical analysis was evaluated by a proportional hazard model (Cox). * and ** denote statistically significant (P value<0.05) and borderline significant (0.05≤P value<0.10) respectively. Variables with P value of less than 0.05 in univariate analysis were further examined in multivariate analysis. †: Data were evaluated as continuous variables, and all other data were evaluated as dichotomized variables. AR, androgen receptor; CYC1, cytochrome c1; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; LI, labeling index; pT, pathological T factor; 95% CI, 95% confidence interval.

Fig. 3B) under the same conditions. Similarly, CYC1 expression was decreased in T47D cells transfected with siCYC1-1 (Fig. 4A) or siCYC1-2 (Fig. 4B) both at mRNA and protein levels.

Effects of CYC1 on proliferation activity in breast carcinoma cells were summarized in Figures 3C-E, 4C, D. The number of cells was significantly increased in MCF7 cells transfected with siCYC1-1 from 3 to 4 days after the transfection than the control cells transfected with siCTL (Fig. 3C). A similar tendency was detected in T47D cells under the same condition (Fig. 4C). Transfection of siCYC1-2 also significantly increased cell proliferation both in MCF7 (Fig. 3D) and T47D (Fig. 4D) cells at 4 days after transfection. In contrast, the proliferation activity was significantly decreased in MCF10DCIS.com cells, which is an ER-negative DCIS

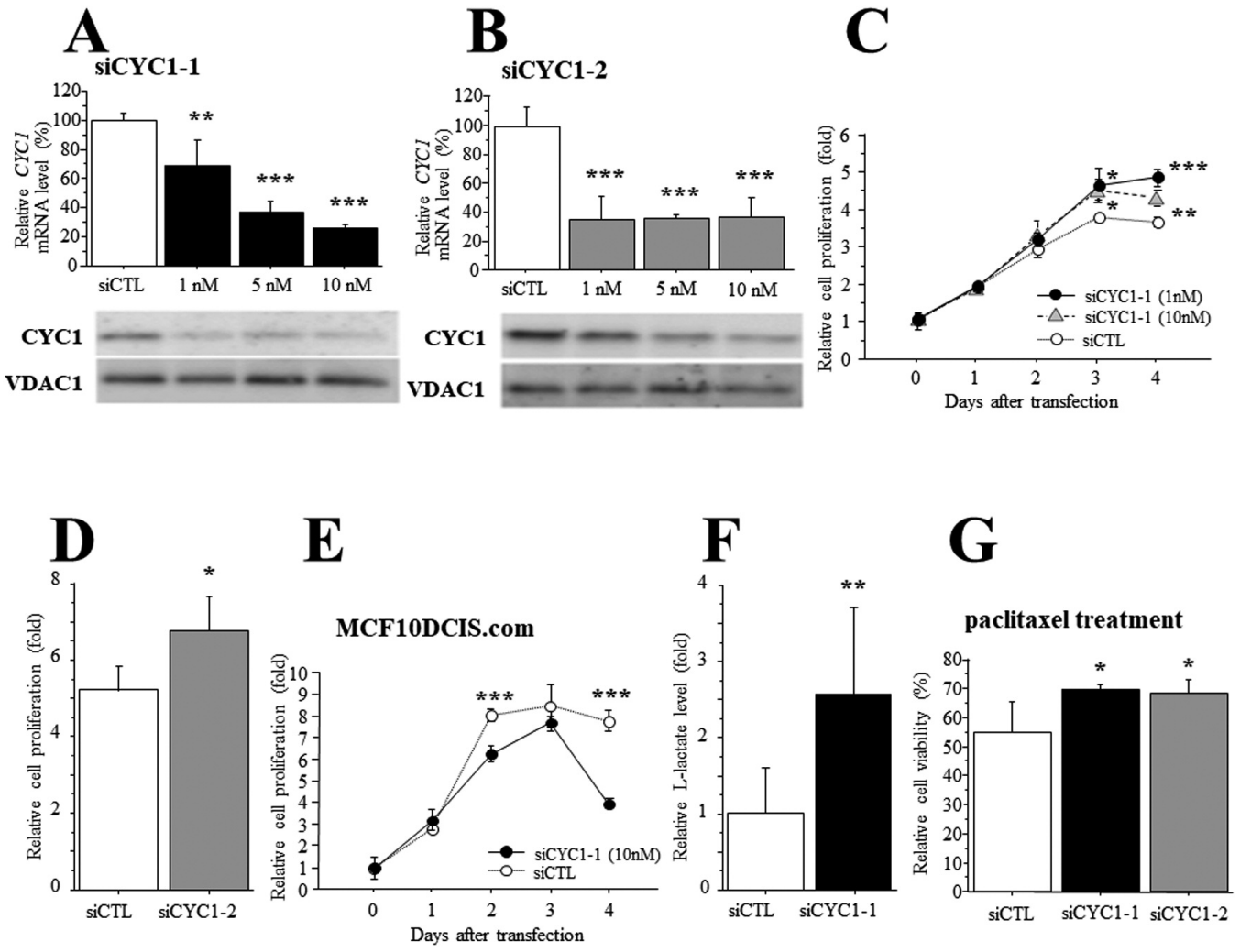

Fig. 3. Effects of CYC1 on proliferation and chemosensitivity in MCF7 cells. **A, B.** Expression level of CYC1 in MCF7 cells transfected with CYC1-specific siRNA. The upper panels show expression of CYC1 mRNA in cells transfected with siCYC1-1 (A; closed bar) and siCYC1-2 (B; gray bar) or negative control siRNA (siCTL, open bar) by real-time PCR. The lower panels summarize the corresponding CYC1 immunoreactivity by immunoblotting. VDAC1 immunoreactivity is shown as an internal control of mitochondrial protein. **C.** Proliferation activity of MCF7 cells transfected with siCYC1-1 (1 nM and 10 nM). Data are summarized as a ratio compared to that at 0 day after treatment. **D.** Proliferation activity of MCF7 cells transfected with siCYC1-2 (10 nM) at 4 days after transfection. Data are presented as a ratio compared to that at 0 day after treatment. **E.** Proliferation activity of MCF10DCIS.com ER-negative DCIS cells transfected with siCYC1-1 (10 nM) summarized as a ratio compared to that at 0 day after treatment. **F.** L-lactate concentration of MCF7 cells transfected with siCYC1-1 (10 nM) at 3 days after transfection. Relative L-lactate level was calculated as the ratio of siCTL (open bar). **G.** Effects of paclitaxel on cell viability of MCF7 according to CYC1 expression. The cells were transfected with siCYC1-1 (closed bar), siCYC1-2 (gray bar) or siCTL (open bar) and treated with paclitaxel (3 nM for 2 days). Relative cell viability was calculated as the ratio of that in the nontreatment cells using the WST-8 assay. In all figures, data were presented as mean ± SD (n=3). *; P<0.05, **; P<0.01 and ***; P<0.001.

cell line, transfected with siCYC1-1 at 2 and 4 days after the transfection as compared to the control cells transfected with siCTL (Fig. 3E), as reported previously (Chisiki et al., 2017).

To further assess the effect of CYC1 on glycolysis, we measured the extracellular L-lactate concentration of siCYC1-1 transfected MCF7 and T47D cells. Relative L-lactate level was significantly increased in both MCF7 (Fig. 3F) and T47D cells (Fig. 4E).

We also examined the effects of CYC1 on chemosensitivity in ER-positive breast carcinoma cells using paclitaxel, which is one of the most common chemotherapeutic agents used in the management of breast cancer patients (Rajappa et al., 2018). Relative cell viability was significantly higher both in MCF7 (Fig. 3G) and T47D (Fig. 4F) cells transfected with siCYC1-1 and siCYC1-2 compared to the cells transfected with siCTL after the paclitaxel treatment.

Discussion

In this study, CYC1 immunoreactivity was detected

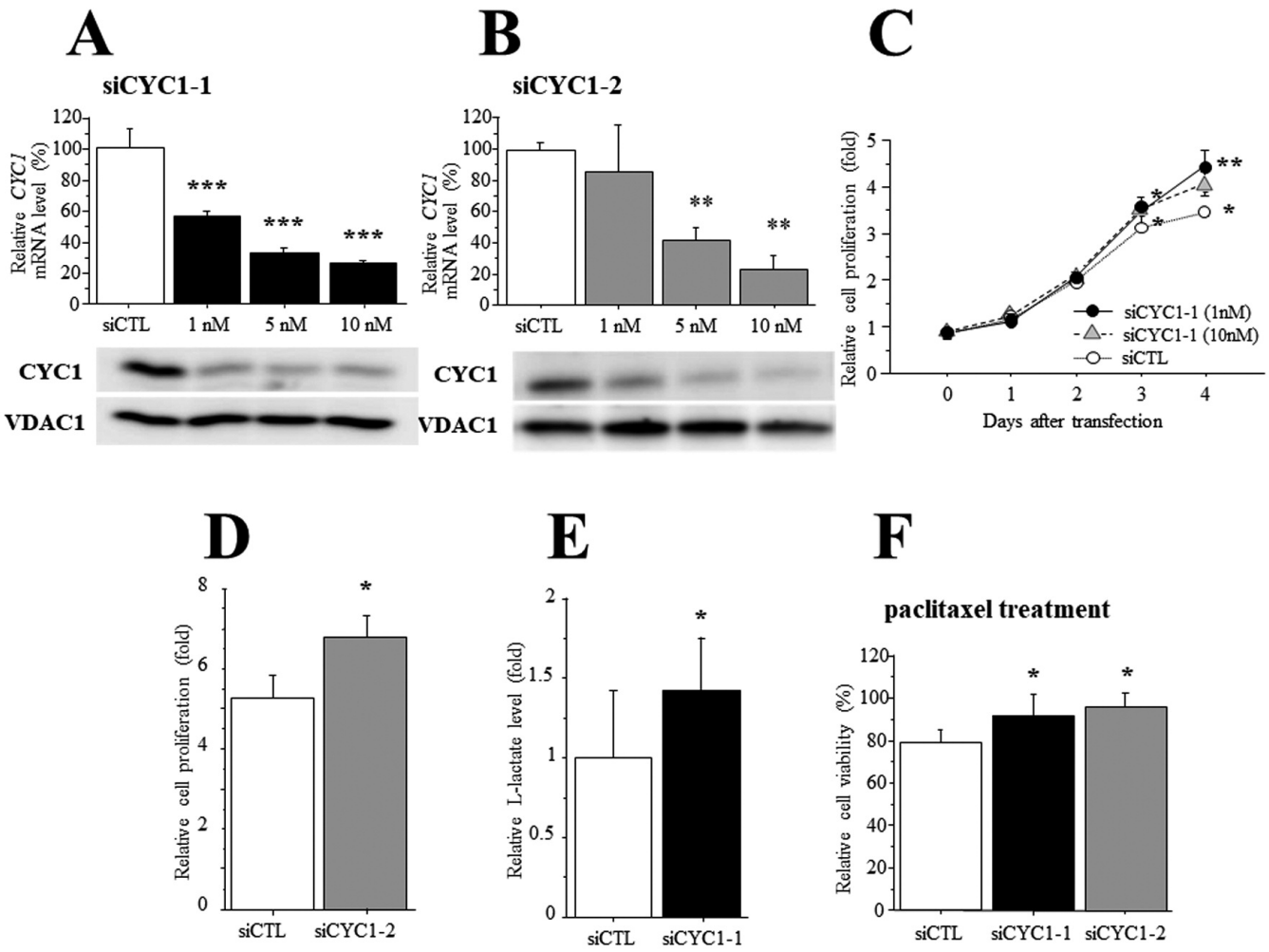

Fig. 4. Effects of CYC1 on proliferation and chemosensitivity in T47D cells. **A, B.** Expression level of CYC1 in T47D cells transfected with CYC1-specific siRNA. The upper panels show expression of CYC1 mRNA in cells transfected with siCYC1-1 (A; closed bar) and siCYC1-2 (B; gray bar) or negative control siRNA (siCTL, open bar) by real-time PCR. The lower panels summarize the corresponding CYC1 immunoreactivity by immunoblotting. VDAC1 immunoreactivity is shown as an internal control of mitochondrial protein. **C.** Proliferation activity of T47D cells transfected with siCYC1-1 (1 nM and 10 nM). Data are summarized as a ratio compared to that at 0 day after treatment. **D.** Proliferation activity of T47D cells transfected with siCYC1-2 (10 nM) at 4 days after transfection. Data are presented as a ratio compared to that at 0 day after treatment. **E.** L-lactate concentration of T47D cells transfected with siCYC1-1 (10 nM) at 3 days after transfection. Relative L-lactate level was calculated as the ratio of siCTL (open bar). **F.** Effects of paclitaxel on cell viability of T47D according to CYC1 expression. The cells were transfected with siCYC1-1 (closed bar), siCYC1-2 (gray bar) or siCTL (open bar) and treated with paclitaxel (0.3 nM 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 were presented as mean ± SD (n=3). *; P<0.05, **; P<0.01 and ***; P<0.001.

in 47% of ER-positive invasive breast carcinomas and 30% of ER-negative invasive breast carcinomas respectively. On the other hand, it was much weaker in morphologically normal mammary glands compared to carcinoma cells. Recently, Han et al. (2016) reported that immunohistochemical score of CYC1 was significantly increased in breast carcinoma (n=26) compared to the benign tumor, and Chishiki et al. (2017) showed that CYC1 was immunolocalized in 40% of DCIS cases. Therefore, it is suggested that CYC1 is overexpressed in many cases of breast cancer from the early stage and plays some roles in progression. In our study, CYC1 immunoreactivity was marginally significant (P=0.070) with ER status in invasive carcinoma, which may be partly because estrogen induced CYC1 expression *via* nuclear respiratory factor 1 in breast carcinoma cells (Ivanova et al., 2011).

Our present study demonstrated that CYC1 immunoreactivity was inversely associated with Ki67 LI in the ER-positive invasive breast carcinoma and immunohistochemical CYC1 status was a significantly favorable prognostic factor for recurrence and breast cancer-specific survival of the patients. Moreover, in our *in vitro* experiments, *CYC1* knockdown increased cell proliferation and glycolytic activity in MCF7 and T47D cells. Previously, Chishiki et al. (2017) showed that CYC1 increased cell proliferation through the elevated oxidative phosphorylation (OXPHOS) activity in DCIS cells. Cellular energy is generated through OXPHOS and glycolysis in carcinoma cells, and these are active inside carcinoma cells at various rates (Guppy et al., 2002). Glycolysis can provide precursors for biomolecules necessary for cell proliferation (Księżakowska-Łakoma et al., 2014; Yang et al., 2016), although its efficiency of ATP synthesis is much lower than that in OXPHOS, and it is considered that proliferating carcinoma cells preferentially use glycolysis rather than OXPHOS for energy production (i.e., Warburg effect). Indeed, Owens et al. (2011) reported that mitochondrial OXPHOS complex III was reduced significantly in the metastatic and aggressive breast cancer cell lines, and Sato-Tadano et al. (2013) reported that immunoreactivity of hexokinase II, which plays a rate-limiting step of glycolysis, was detected in 44% of invasive breast carcinoma and it was the potent worse prognostic factor. Taken together with our present results and these previous reports, it is suggested that the OXPHOS pathway does not necessarily play a pivotal role in the progression of ER-positive invasive breast carcinoma, different from glycolysis, and CYC1 immunoreactivity is associated with a better prognostic phenotype of the ER-positive invasive breast carcinoma. On the other hand, HER2 status, lymph node metastasis and histological grade did not independently predict the recurrence or mortality of patients. This may be partly due to the fact that we focused on only ER-positive breast cancer patients and these parameters do not necessarily serve as predictive factors in ER-positive cases (Nielsen et al., 2010). In addition, CYC1 and these parameters may have weakened each others predictive power, because of relatively smaller sample size.

Han et al. (2016) recently showed that CYC1 predicted poor prognosis in patients with breast cancer using a public microarray database and that CYC1 induced proliferative and metastatic properties in ER-negative breast carcinoma cells (MDA-MB-231 and MDA-MB-435S). These results seem to be inconsistent with our present findings, but it may be partly due to the difference of ER status focused on in these studies. ER-positive and ER-negative breast carcinoma should be biologically distinguished from each other. Although we did not add exogenous estrogens in the media in cell proliferation assay, FBS supplied in the media contains estrogens to some extent. Previously, it has been reported that estrogens modify the mitochondrial dynamics, biogenesis and metabolism in MCF7 cells (Sastre-Serra et al., 2012). On the other hand, reactive oxygen species (ROS) has been reported to inhibit estrogen-dependent growth of MCF7 cells, while mitochondria are a major source of ROS (Felty and Roy, 2005). Considering these previous reports and recent findings, it may be possible that CYC1 is associated with estrogen dependent growth in ER-positive breast carcinoma cells, partially explaining the different roles and clinical significance of CYC1 in ER-positive and ER-negative breast carcinoma. Replication studies with a larger sample set and/or a longer-follow up period are needed to confirm clinical significance of CYC1 immunoreactivity in the breast carcinoma according to ER status. In addition, it is also required to assess in more detail the effect of CYC1 on estrogen dependent or independent proliferation in breast carcinoma cells.

In our study, CYC1 status was significantly associated with better prognosis of ER-positive breast cancer patients who received adjuvant chemotherapy and *in vitro* experiments revealed that CYC1 increased chemosensitivity to paclitaxel in MCF7 and T47D cells. Paclitaxel is one of the most common chemotherapeutic agents used in adjuvant and neoadjuvant chemotherapy and in the management of metastatic breast cancer (Rajappa et al., 2018). Paclitaxel is a microtubule disturbing agent that increases microtubule stability, decreases microtubule disassembly and increases mitotic arrest which precedes apoptosis (Horwitz, 1994). An association between CYC1 and chemosensitivity has not been reported. Previously, Morse et al. (2005) reported that treatment of MCF7 cells with other taxans, docetaxel, caused non-apoptotic cell death through mitotic catastrophe, although MCF7 cells are partially resistant to apoptosis due to elevation in the apoptosis-inhibiting product Bcl-2 and caspase-3 defects. Considering that mitochondria regulate the cell cycle and proliferation (Antico Arciuch et al., 2012), CYC1 may, at least in part, involve chemosensitivity of breast carcinoma through the regulation of cell cycle. Because CYC1 is associated with a better prognostic phenotype of ER-positive breast carcinoma through possibly regulating a variety of biological functions, such as

energy production, cell cycle and chemosensitivity, as described in this section, CYC1 immunoreactivity could become a potent favorable prognostic marker and predict the efficacy of chemotherapy, especially paclitaxel in ER-positive breast carcinoma. Further examinations are required to clarify the molecular mechanism of CYC1 in breast carcinoma according to the ER status.

In summary, we immunolocalized CYC1 immunoreactivity in the invasive breast carcinoma, and it was positive in 47% of ER-positive cases and 30% of ER-negative cases respectively. Immunohistochemical CYC1 status was inversely associated with Ki67 in the ER-positive cases, and it was a significantly favorable prognostic factor for both disease-free and breast cancer-specific survival. On the other hand, no significant association was detected between CYC1 status and clinicopathological factors in the ER-negative cases. Following *in vitro* experiments revealed that *CYC1* knockdown increased cell proliferation, glycolytic activity and chemosensitivity to paclitaxel in ER-positive breast carcinoma cells. These results suggest that CYC1 is associated with a better prognostic phenotype of ER-positive breast carcinoma through possibly regulating a variety of biological functions, such as energy production, cell cycle, and chemosensitivity, and CYC1 immunoreactivity becomes a potent favorable prognostic marker in ER-positive breast cancer patients.

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References

Antico Arciuch V.G., Elguero M.E., Poderoso J.J. and Carreras M.C. (2012). Mitochondrial regulation of cell cycle and proliferation. Antioxid. Redox Signal. 16, 1150-1180.

Brouckaert O., Laenen A., Vanderhaegen J., Wildiers H., Leunen K., Amant F., Berteloot P., Smeets A., Paridaens R., Christiaens MR., Floris G., Moerman P., Van Limbergen E., Peeters S., Weltens C., Vergote I. and Neven P. (2012). Applying the 2011 St Gallen panel of prognostic markers on a large single hospital cohort of consecutively treated primary operable breast cancers. Ann. Oncol. 23, 2578-2584.

Chishiki M., Takagi K., Sato A., Miki Y., Yamamoto Y., Ebata A., Shibahara Y., Watanabe M., Ishida T., Sasano H. and Suzuki T. (2017). Cytochrome c1 in ductal carcinoma *in situ* of breast associated with proliferation and comedo necrosis. Cancer Sci. 108, 1510-1519.

Clayton DA. and Shadel GS. (2014). Isolation of mitochondria from tissue culture cells. Cold Spring Harb. Protoc. 2014, pdb.prot080002.

Djalalov S., Beca J., Amir E., Krahn M., Trudeau ME. and Hoch J.S. (2015). Economic evaluation of hormonal therapies for postmenopausal women with estrogen receptor-positive early breast cancer in Canada. Curr. Oncol. 22, 84-96.

Duncan A.M., Ozawa T., Suzuki H. and Rozen R. (1994). Assignment of the gene for the cytochrome c1 subunit of the mitochondrial cytochrome bc1 complex (CYC1) to human chromosome 8q24.3. Genomics 19, 400-401.

Fang W., Li X., Jiang Q., Liu Z., Yang H., Wang S., Xie S., Liu Q., Liu T., Huang J., Xie W., Li Z., Zhao Y., Wang E., Marincola F.M. and Yao K. (2008). Transcriptional patterns, biomarkers and pathways characterizing nasopharyngeal carcinoma of Southern China. J. Transl. Med. 6, 32

Felty Q. and Roy D. (2005). Mitochondrial signals to nucleus regulate estrogen-induced cell growth. Med. Hypotheses 64, 133-141.

Fisher B. and Anderson S.J. (2010). The breast cancer alternative hypothesis: is there evidence to justify replacing it? J. Clin. Oncol. 28, 366-374.

Guppy M., Leedman P., Zu X. and Russell V. (2002). Contribution by different fuels and metabolic pathways to the total ATP turnover of proliferating MCF-7 breast cancer cells. Biochem. J. 364, 309-315.

Hammond M.E., Hayes D.F., Dowsett M., Allred D.C., Hagerty K.L., Badve S., Fitzgibbons P.L., Francis G., Goldstein N.S., Hayes M., Hicks D.G., Lester S., Love R., Mangu P.B., McShane L., Miller K., Osborne C.K., Paik S., Perlmutter J., Rhodes A., Sasano H., Schwartz J.N., Sweep F.C., Taube S., Torlakovic E.E., Valenstein P., Viale G., Visscher D., Wheeler T., Williams R.B., Wittliff J.L. and Wolff A.C; American Society of Clinical Oncology; College of American Pathologists. (2010). American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer (unabridged version). Arch. Pathol. Lab. Med. 134, e48-72.

Han Y., Sun S., Zhao M., Zhang Z., Gong S., Gao P., Liu J., Zhou J., Ma D., Gao Q. and Wu P. (2016). CYC1 predicts poor prognosis in patients with breast cancer. Dis. Markers 2016, 3528064.

Horwitz SB. (1994). Taxol (paclitaxel): mechanisms of action. Ann. Oncol. 5 (Suppl 6), S3-6.

Ivanova MM., Luken K.H., Zimmer A.S., Lenzo F.L., Smith R.J., Arteel M.W., Kollenberg T.J., Mattingly K.A. and Klinge C.M. (2011). Tamoxifen increases nuclear respiratory factor 1 transcription by activating estrogen receptor beta and AP-1 recruitment to adjacent promoter binding sites. FASEB J. 25, 1402-1416.

Księżakowska-Łakoma K., Żyła M. and Wilczyński J.R. (2014). Mitochondrial dysfunction in cancer. Prz. Menopauzalny. 13, 136-144.

Li G., Fu D., Liang W., Fan L., Chen K., Shan L., Hu S., Ma X., Zhou K. and Cheng B. (2014). CYC1 silencing sensitizes osteosarcoma cells to TRAIL-induced apoptosis. Cell. Physiol. Biochem. 34, 2070-2080.

Miller F.R., Santner S.J., Tait L. and Dawson P.J. (2000). MCF10DCIS.com xenograft model of human comedo ductal carcinoma *in situ*. J. Natl. Cancer Inst. 92, 1185-1186.

Minemura H., Takagi K., Sato A., Takahashi H., Miki Y., Shibahara Y., Watanabe M., Ishida T., Sasano H. and Suzuki T. (2016). CITED2 in breast carcinoma as a potent prognostic predictor associated with proliferation, migration and chemoresistance. Cancer Sci. 107, 1898-1908.

Morse D.L., Gray H., Payne C.M. and Gillies R.J. (2005). Docetaxel induces cell death through mitotic catastrophe in human breast

cancer cells. Mol. Cancer Ther. 4, 1495-1504.

Nielsen T.O., Parker J.S., Leung S., Voduc D., Ebbert M., Vickery T., Davies S.R., Snider J., Stijleman I.J., Reed J., Cheang M.C., Mardis E.R., Perou C.M., Bernard P.S. and Ellis M.J. (2010). A comparison of PAM50 intrinsic subtyping with immunohistochemistry and clinical prognostic factors in tamoxifen-treated estrogen receptor-positive breast cancer. Clin. Cancer Res. 16, 5222-5232.

Owens K.M., Kulawiec M., Desouki M.M., Vanniarajan A. and Singh K.K. (2011). Impaired OXPHOS complex III in breast cancer. PLoS One 6, e23846.

Rajappa S., Joshi A., Doval D.C., Batra U., Rajendranath R., Deo A., Biswas G., Bajpai P., Tilak T.V.S., Kane S., Kumar K., Kumar M., Talele A.D., Devde P., Gupta A., Joshi N., Sejpal J., Bunger D. and Khan M. (2018). Novel formulations of docetaxel, paclitaxel and doxorubicin in the management of metastatic breast cancer. Oncol. Lett. 16, 3757-3769.

Sastre-Serra J., Nadal-Serrano M., Pons D.G., Roca P. and Oliver J. (2012). Mitochondrial dynamics is affected by 17β-estradiol in the MCF-7 breast cancer cell line. Effects on fusion and fission related genes. Int. J. Biochem. Cell Biol. 44, 1901-1905

Sato-Tadano A., Suzuki T., Amari M., Takagi K., Miki Y., Tamaki K., Watanabe M., Ishida T., Sasano H and Ohuchi N. (2013). Hexokinase II in breast carcinoma: a potent prognostic factor associated with hypoxia-inducible factor-1α and Ki-67. Cancer Sci. 104, 1380-1388.

Tevaarwerk A.J., Gray R.J., Schneider B.P., Smith M.L., Wagner LI., Fetting J.H., Davidson N., Goldstein L.J., Miller K.D. and Sparano J.A. (2013). Survival in patients with metastatic recurrent breast cancer after adjuvant chemotherapy: little evidence of improvement over the past 30 years. Cancer 119, 1140-1148.

Yang Y., Karakhanova S., Hartwig W., D'Haese J.G., Philippov P.P., Werner J. and Bazhin A.V. (2016). Mitochondria and mitochondrial ROS in cancer: Novel targets for anticancer therapy. J. Cell. Physiol. 231, 2570-2581.

Zhu Y., Li M., Wang X., Jin H., Liu S., Xu J. and Chen Q. (2012). Caspase cleavage of cytochrome c1 disrupts mitochondrial function and enhances cytochrome c release. Cell Res. 22, 127-141.

Zoli W., Ulivi P., Tesei A., Fabbri F., Rosetti M., Maltoni R., Giunchi D.C., Ricotti L., Brigliadori G., Vannini I. and Amadori D. (2005). Addition of 5-fluorouracil to doxorubicin-paclitaxel sequence increases caspase-dependent apoptosis in breast cancer cell lines. Breast Cancer Res. 7, R681-689.

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