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Original Article

Quercetin Enhances Tamoxifen-Induced Antitumor Activity Through Down-Regulation of Cyclin E2 Expression in Drug-Resistant Human Breast Cancer Cells

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Abstract.

Quercetin is found in plant tissues known as flavonoids, and contains properties that act as a chemopreventive or chemosensitizer agent against human breast cancer. However, the oncogenic molecule inhibited by quercetin treatment has still not been identified in human breast cancer cells. The cyclin E2 (CCNE2) mRNA levels were demonstrated to be higher in tumor cells (3.55-fold, $*P = 0.005$) than in normal paired tissue samples, using real-time reverse-transcriptase polymerase chain reaction (RT-PCR) analysis (n = 213). We further demonstrated that relatively higher levels of CCNE2 protein expression were detected in the tamoxifen-resistant (TAM-R) MCF-7 cells. The results showed that CCNE2 protein expression levels were specifically inhibited in quercetin-treated (5 μM) TAM-R cells, either in the presence or absence of 4-OH-TAM (100 nM). Additionally, it was noted that combination treatment with 4-OH-TAM and quercetin significantly sensitized TAM-R cells to 4-OH-TAM. This study suggests that quercetin can be used as a chemosensitizer by targeting CCNE2 expression levels, which may be a novel strategy to overcome TAM resistance in breast cancer patients.

Keywords **:** quercetin, CCNE2, breast cancer, tamoxifen, cell cycle

原著論文

槲黃素透過細胞週期蛋白 Cyclin E2 的抑制作用增強對 Tamoxifen 抗藥性乳癌細胞之抗癌作用

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中文摘要

目的：槲黃素(quercetin)可以在許多植物組織中發現，是一種類黃酮成分。槲黃素的抗乳癌作用主要是化學預防與作為化學治療藥物的增強作用。至於它在乳癌細胞內真正作用於何種致癌蛋白並未有任何報導。

材料方法：本實驗利用即時反轉錄酶鏈鎖反應(real-time reverse-transcriptase polymerase chain reaction)分析細胞週期蛋白 cyclin E2 (CCNE2) mRNA 在 213 位乳癌病人身上的表現情況，並利用 MCF7 乳癌細胞株作為研究模式，探討槲黃素之抑制 CCNE2 的作用機制。

結果：證實 CCNE2 mRNA 在癌組織中的表現明顯高於正常組織約 3.55 倍 (*P = 0.005)。證實 CCNE2 蛋白在 Tamoxifen 抗藥性 MCF-7 細胞株(TAM-R)中，CCNE2 高表現。此種 TAM-R 細胞株 CCNE2 高表現可以被槲黃素(5 μM)所抑制。合併處理槲黃素與 Tamoxifen 可以使抗藥性 MCF-7 細胞株對藥物的敏感度增加。

結論：槲黃素有潛力與 Tamoxifen 作為合併使用，可以使抗藥性 MCF-7 細胞株對藥物的敏感度增加，並增加化療效果解決乳癌病人抗藥性的問題。

關鍵字: 槲黃素、細胞週期蛋白 cyclin E2、乳癌、諾瓦得士錠、細胞週期

INTRODUCTION

Quercetin is a flavonoid which is widely distributed in nature, and routinely found in a variety of vegetables, fruits, leaves and assorted grains. Foods containing quercetin, presented in milligrams per 100 grams of edible portion (shown as numerals in parentheses), include brewed, black or green tea (Camellia sinensis, about 2), Red Delicious apples (4), cow peas (11), sweet potato (10), kale (23), watercress (30), red onion (32, higher concentrations of quercetin occur in the outermost rings and in the part closest to the root, the latter being the part of the plant with the highest concentration)[6], broccoli (3), black plums (12) and a number of berries, including cultivated blueberry (8), bilberry (3), lingonberry (13), cranberry (15), chokeberry (19), rowanberry (7), sea buckthorn berry (8), crowberry (5) and the fruit of the prickly pear cactus (5). The best dietary sources of quercetin are celery, green peppers, carrots, olive oil and artichokes, and it is also found in many commonly used herbs such as thyme, parsley, and oregano. Recent studies have demonstrated that quercetin can also be used as a chemosensitizer to improve the therapeutic effects of some anti-tumor drugs in breast cancer cells [1]. The results demonstrated that quercetin exerts its antitumor

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Table 1. Anti-tumor activities of quercetin in human cancer cells

Combination treatment as a chemosensitizer in cancer therapy	In vitro [a] (μM)	In vivo [b] (mg/Kg)	Mechanisms of antitumor activity	Cancer cell types
Trichostatin A	2-5	10	Apoptosis, cell growth arrest [a]	A549
Doxorubicin	1-10	100	Inhibition of tumor cell growth [c]	SMMC7721, QGY7701
Daunorubicin	1-100		Apoptosis [d]	EPG85-257P
17β-estradiol	1		Pro-apoptosis, cell cycle arrest [e]	HeLa and DLD-1 cells
TNF-related apoptosis-inducing ligand (TRAIL)	10-100		Apoptosis [i]	VAL, RL and SUDHL4
Hypoxia	25-100	50	Apoptosis [j],	HCT116
Nelfinavir, bortezomib	10-60	20	Apoptosis, proteasome inhibition [k]	MCF-7, MDA-MB-453
IL-6	1-50		Cell growth inhibition, cell migration [l]	U87 and T98G
Luteolin	10-40		Anti-metastatic activity [m]	A549, A431
Luteolin	0.1-10		Apoptosis, cell growth arrest [n],	MCF-7, MDA-MB-231, BT-483
Vascular endothelial growth factor	10-40	20	Cell growth inhibition [o]	HUVEC
Single treatment as a potential cancer therapy agent	**In vitro [a] (μM)**	**In vivo [b] (mg/Kg)**	**Mechanisms of antitumor activity**	**Cancer cell types**
	10-30		Apoptosis, cell growth arrest [g]	SCC25
	5 -50		Cell growth arrest, apoptosis [h]	HepG2
	25-200		Apoptosis [p]	HL-60

[a]: In vivo study [10], [b]: in vitro study [36], [c]: p53-mediated antitumor signals [37], [d]: inhibition of multi-drug resistant [38], [e]: mimic cyclin E2 effects [38], [f]: inhibition of the epithelial-mesenchymal transition [39-41], [g]: cancer stem cells targeting [32], [h]: reduction of nuclear factor erythroid 2-related factor 2 (Nrf2) signaling [33], [i]: mitochondria-mediated signaling pathways [42], [j]: AMP-activated protein kinase inhibition [43], [k]: inhibition of the autophagy-controlling mTOR activity [44], [l]: JAK/STAT3 signaling pathway inhibition [45], [m,n]: against multidrug resistant (MDR) cancer cells [40,46], [o]: anti-VEGF-induced angiogenesis [3], [p]: PI3K/AKT signaling inhibition [47]

effects through mechanisms such as apoptosis induction [2], anti-angiogenesis [3], prevention of cell metastasis [4] and arrest of cell growth proliferation [5]. Some scientific studies have attributed a number of antitumor benefits to the consumption of quercetin, specifically, combining anti-tumor agent treatments with quercetin [6]. Many studies have tried to explain the antitumor effect of quercetin by identifying specific molecules that respond to quercetin. For example, we have demonstrated that the combined treatment of luteolin and quercetin inhibited the nicotinic receptor to block smoking-induced breast cancer formation [7]. Other molecules such as aromatase [8], which is the key enzyme in estrogen biosynthesis [8], multidrug resistant (MDR) genes [9], and the up-regulation of tumor suppressor genes [10] were identified as intracellular targets for quercetin treatment. Research on the possible health benefits of these substances is still ongoing and not yet conclusive, but quercetin supplements should prove to be a novel strategy for cancer therapy (Table 1).

In mammalian cells, there are two E-type cyclins, cyclin E1 and E2 (collectively referred to as cyclin E). The predominant function of cyclin E is believed to be Cdk2 activation and the consequent effects on cell cycle progression and DNA replication, because both E-type cyclins accelerate the G1-to-S-phase transition. An earlier study demonstrated that cyclin E1 is overexpressed in a mouse mammary gland model at a low frequency (~12%) and with a long latency period (8 to 13 months) [11], although tumourigenesis is enhanced by cooperation with other oncogenic events, such as p53 inactivation [12]. These studies support the association between cyclin E1 and breast cancer formation. Interestingly, CCNE2 has never been independently examined in breast tumorigenesis models. In contrast with the apparent redundancy between these two E-type cyclins in many research papers, clinical studies have suggested that cyclin E1 and E2 may have distinct roles in breast cancer formation, with CCNE2 being particularly important in estrogen receptor (ER)-positive cancers. To evaluate the prognostic value of cyclin E, a quantitative real-time PCR assay was performed using 635 cases of lymph node-negative breast cancer [13]. The results indicated that CCNE2 mRNA is associated with poor outcome only in ER-positive cancers; however, the cyclin E1 mRNA is prognostic for both ER-positive and ER-negative cancers [13]. Another study showed that CCNE2 mRNA is significantly associated with outcome in systemically untreated patients, but not in TAM-treated patients [14]. Such results indicated that a high level of CCNE2 is a predictor of poor outcome in breast cancer patients, especially in the context of estrogen action.

A previous study surveyed three microarray breast tumor datasets with ER-positive status that had received only TAM as adjuvant treatment [15]. Interestingly, this study found that there were only four genes in common across all three sets of data in which the CCNE2 are overexpressed in TAM-resistant tumors. Compared with cyclin E1, CCNE2 was significantly induced by estrogen, which then leads to the formation of a substantial amount of the CCNE2/Cdk2 complex accompanied by decreased binding of Cdk2 inhibitors (such as p21/waf1/Cip1 and p27Kip1) [16]. Ectopic expression of cyclin E1 reduces acute sensitivity to anti-estrogen arrest in MCF-7 cells [12]. The overexpression of either cyclin E1 or E2 is capable of shortening the G1 phase and increasing overall breast cancer cell proliferation [17]. Ablation of cyclin E1 or CCNE2 attenuates estrogen-induced MCF-7 cell proliferation [16]. These results indicate that overexpression of CCNE2 may play a positive role in estrogen-induced cell proliferation and act against anti-estrogen-induced cell death effects, causing anti-estrogen drug resistance.

Estrogen stimulates proliferation through the modulation of a panel of cell cycle proteins, resulting in rapid entry into the cell cycle from quiescence. In breast cancer cells, the estrogen-induced cell growth effects converge on the activation of cyclin E-Cdk2,

which is essential for cell cycle progression. Functional ablation of Cdk2 activity causes anti-estrogen resistance in human breast cancer cells [18]. Cyclin E1 and E2 are well-known E2F targets. A promoter-binding activity assay demonstrated that E2F1 (and not E2F2-5) is recruited to both E1 and E2 promoters after estrogen stimulation; however, only CCNE2 is significantly up-regulated [16]. Microarray analysis has identified CCNE2 as a strong estrogen-responsive gene [19,20].

In this study, we found that CCNE2 was detected at a higher level (3.55-fold) in 213 cases of breast tumor tissue samples compared with normal tissue. We further demonstrated that higher expression levels of CCNE2 in tumor tissues were significantly associated with luminal type A (ER+/PR+/Her2-) tumors. It would appear that a combination treatment with 4-OH-TAM (100 nM) and quercetin (5 μM) significantly sensitized TAM-R cells to 4-OH-TAM. This study provides molecular evidence suggesting that targeting CCNE2 expression through food components, such as quercetin, may be a novel strategy to overcome TAM resistance in breast cancer patients.

MATERIALS AND METHODS

Chemicals

Quercetin (with 99.5% purity) and ICI 182780 were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA). Those chemicals used in this study were dissolved in dimethyl sulfoxide (DMSO) as described in our previous studies [21,22].

Cell Culture and Patient Samples

Human breast tumor samples were obtained as anonymous specimens from the Taipei Medical University Hospital and Cathay General Hospital, Taipei, following a protocol approved by the Institutional Review Board (P950012). This study was performed using 213 patients already present in our database and involved no increased risk for the subjects. Informed consent was obtained from all patients for this study, and the Declaration of Helsinki recommendations for biomedical research involving human subjects were properly followed. Human mammary gland adenocarcinomas of different ER status (ER+ cells: MCF-7, BT-483, BT-474; ER- cells: MDA-MB-231) and an ER-normal human mammary epithelial cell line (MCF-10A) were purchased from the American Type Culture Collection (Middlesex TW110LY, United Kingdom). The cells were grown in the recommended medium (Mediatech, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS; Invitrogen, Carlsbad, CA, USA) and penicillin-streptomycin antibiotics (Mediatech). TAM-R cells derived from MCF-7 cells were continuously cultured in a medium containing 10^{-7} mol/L 4-hydroxytamoxifen (4-OH-TAM, Sigma-Aldrich, Dorset, UK) [23,24]. The medium for the matched control cells contained 0.1% ethanol (EtOH). Cell proliferation and viability were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay.

RNA Isolation and Real-Time Polymerase Chain Reaction (PCR)

Total RNA was isolated from human cell lines, and breast tumor tissue samples were acquired directly from patients using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol [25]. The primers used were as follows: sense (CCNE2), GCTATGCTGGAGGAAGTAAAT; anti-sense (CCNE2), CAGTGATACCAGTTCTACCC; sense (GUS), AAA CAGCCCGTTTACTTGAG; and anti-sense (GUS), AGTGTTCCCTGCTAGAATAGATG. Real-time PCR reactions were performed using a LightCycler thermocycler (Roche Molecular Biochemicals, Mannheim, Germany). The CCNE2 mRNA fluorescence intensity was measured and normalized to β-glucuronidase (GUS) expression levels using the Roche LightCycler Software, version 4 [26].

Protein Extraction, Western Blotting Analysis, and Antibodies

To examine protein expression levels, human breast tumor cells were thawed in lysis buffer that contained protease inhibitors, and protein (50 μg) from each sample, separated on a 12% SDS-polyacrylamide gel, transferred, and analyzed by Western blotting method. Primary (anti-cyclin E1 and anti-α-tubulin) and secondary (alkaline phosphatase-coupled anti-mouse and anti-rabbit IgG) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA. USA). The anti-CCNE2 antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). The α-tubulin expression levels were used to control for equal protein loading.

Steroid Hormone Treatments

Exponentially proliferating cells were treated for 48 h with 1 x 10^{-8} M ICI 182780 to induce quiescence. The cells were subsequently stimulated with 1 x 10^{-7} M 17β-estradiol [3,17β-dihydroxy-1,3,5(10)-estratriene; (Sigma, Castle Hill, NSW, Australia)] and collected as indicated below. Steroid hormones and antagonists were dissolved in EtOH at a 1,000-fold final concentration, and control cultures received EtOH vehicle to the same final concentration [16].

Cell Proliferation and Flow Cytometry Analyses

The percentage of cells in the S phase of the cell cycle was measured using flow cytometric analysis of propidium iodide-stained, EtOH-fixed cells. The population of nuclei in each phase of the cell cycle was determined using established CellFIT DNA analysis software (Becton Dickenson, San Jose, CA, USA). Cell proliferation was also assessed using hemocytometer cell counts or AlamarBlue (Invitrogen) [27].

Statistical Methods

All data are expressed as the mean ± SD. Unless stated otherwise, the error bars in the figures represent the SD from at least three experiments. The fold ratios of CCNE2 mRNA expression levels in tumor vs. normal samples were compared with regard to various clinical markers using Scheffe's test. The number of paired samples at each stage was analyzed with an overall nonparametric test (Kruskal-Wallis test). Multiple comparisons were assessed using the Mann-Whitney test. All *P*-values are two-sided. Significant differences in the tumor cell viability and relative S-phase cell cycle value were analyzed using the Kruskal-Wallis (nonparametric) test, and each pairwise comparison was performed using the Mann-Whitney test. All statistical comparisons were performed using SigmaPlot graphing software (San Jose, CA, USA) and SPSS Version 11.0.0 (SPSS, Chicago, IL, USA). A *P*-value of <0.05 was considered significant, and all statistical tests were two-sided.

RESULTS

In this study, several types of human breast cancer cell lines were examined for CCNE2 expression (Figure 1A). The results showed that higher protein expression levels of CCNE2 were predominantly detected in three breast cancer cell lines, MCF-7, BT-474, and BT-483. In contrast, CCNE2 protein was expressed in normal breast epithelial cells (MCF-10A) at a relatively lower level. To ascertain whether CCNE2 overexpression occurs in the breast tumor tissue of Asian breast cancer patients, CCNE2 mRNA levels were examined in tumor/normal paired tissue samples using real-time RT-PCR analysis (n = 213; Figure 1B). Based on the real-time PCR results, the cases were divided according to their CCNE2 mRNA expression levels (Figure 1B). The mean level of CCNE2 expression in the tumor cells was 3.55-fold greater than in the normal cells (Figure 1B; $*P = 0.005$). We further evaluated the CCNE2 expression levels in the tumor/normal paired samples, and the association with ER, PR, and Her2 expression status. Interestingly, we found that overexpression of CCNE2 mRNA was preferentially detected in ER+ or PR+ tumor tissues

Table 2. Bivariate analysis of prognostic factors and CCNE2 expression

Factors	CCNE2 N>T			CCNE2 T>N		
	No. of patients	§mean±se	P value	No. of patients	§mean±se	P value
Age			.945			.277
<50yr	45	5.4±0.9		45	9.8±1.6	
≧50yr	58	5.5±0.8		53	7.7±1.2	
Size of tumor			.631			.161
T0-T1	49	5.2±0.6		41	6.0±1.1	
T2	52	5.5±1.0		49	11.3±1.6	
T3	5	4.9±1.4		9	6.0±2.6	
T4	1	10.4		none		
Nodal status			0.291			.957
N0	51	5.8±0.9		55	7.8±1.3	
N1	32	5.9±1.1		24	10.9±1.9	
N2	12	2.8±0.6		13	10.7±3.3	
N3	12	4.8±1.4		7	3.7±1.0	
Stage of disease			.518			.920
0-I	33	5.1±1.0		31	7.0±1.5	
II	50	6.4±1.0		49	10.2±1.5	
III	22	3.5±0.8		16	7.7±2.4	
IV	2	5.8±4.6		3	3.8±2.1	
ER status			.841			.245
Negative	39	5.5±1.0		38	7.3±1.3	
Positive	68	5.3±0.7		59	9.7±1.43	
PR status			.919			.001*
Negative	52	5.3±0.8		52	5.9±0.9	
Positive	54	5.4±0.8		43	12.4±1.7	
Her-2 status			.558			.392
Negative	56	5.0±0.7		56	10.0±1.4	
Positive	32	5.8±1.3		24	7.9±1.8	
5-year survival			.281			.823
Alive	91	5.3±0.6		80	8.3±1.0	
Dead	6	8.1±2.3		6	9.2±4.7	
Chemotherapy			.315			.721
No	25	5.9±1.4		21	7.0±2.1	
Yes	68	5.5±0.7		63	8.8±1.1	
Radiotherapy			.315			.722
No	69	5.9±0.8		64	8.8±1.2	
Yes	25	4.7±1.2		21	6.8±1.6	
Tamoxifen			.316			.721
No	34	5.4±1.1		38	8.0±1.5	
Yes	60	5.6±0.8		47	8.6±1.3	
Herceptin			.250			.580
No	66	5.8±0.7		49	7.6±1.3	
Yes	7	6.1±3.4		9	11.1±3.1	

*Fold ratios of CCNE2 mRNA expression were determined in normal/tumor or tumor/normal paired samples. Data were analyzed using bivariate analyses. A *P*-value < 0.05 was considered as statistically significant. All *P*-values are two-sided

§ mean: average fold ratio of CCNE2 mRNA expression in each group

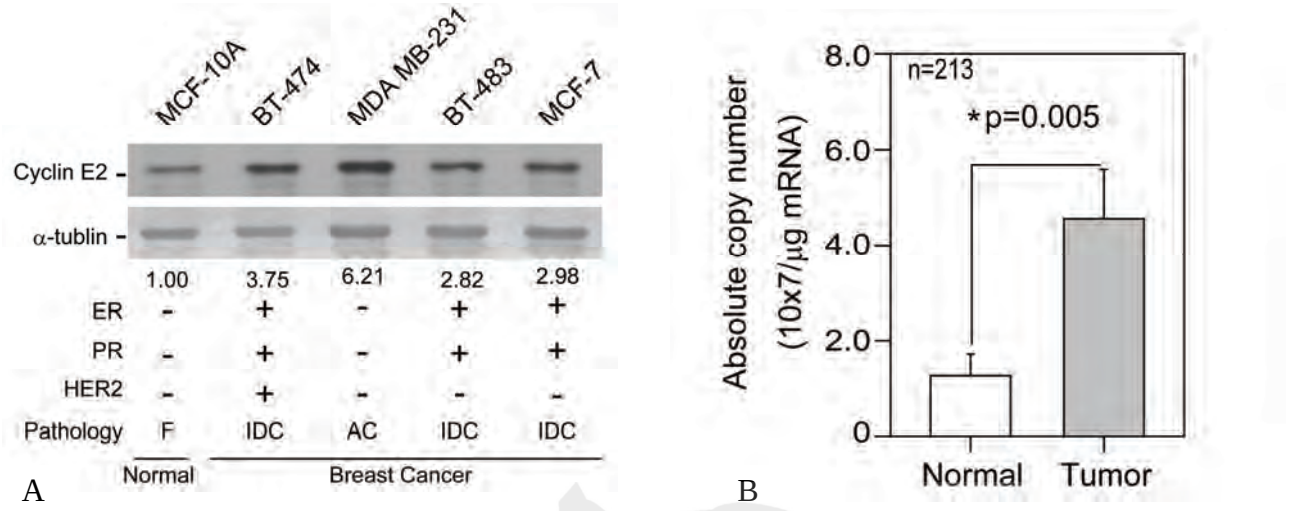

Figure 1. CCNE2 overexpression was preferentially detected in human breast tumor tissues. (A) CCNE2 protein expression profiles in human breast tumor and normal cells were detected using immunoblotting analyses. (B) The CCNE2 mRNA expression profiles in paired human breast tumor and normal tissues (n = 213) were investigated using real-time RT-PCR. The CCNE2 mRNA levels are shown for 213 patient samples in which the expression levels were higher in the tumor than in the normal tissue. The copy numbers (10^7 per μg of mRNA) were calculated from the mean real-time RT-PCR data; the error bars indicate the 95% confidence intervals. The fold ratios of CCNE2 mRNA expression levels detected in tumor versus normal samples with different clinical criteria were compared using the Scheffe's test; all P-values are two-sided

(Table 2). We also found that CCNE2 overexpression was detected preferentially in Her2-negative patients compared with Her2-positive patients, although the results were not significant (Table 2).

Based on these observations, the results indicated that CCNE2 overexpression was preferentially detected in luminal type A ER+/PR+/Her2- patients (n = 34). Luminal type A patients are treated with TAM as a first-line therapeutic agent [28]. Unfortunately, up to 50% of patients with metastatic disease do not respond to first-line treatment with TAM. To test whether CCNE2 overexpression was involved in TAM resistance in human breast cancer cells, the protein expression levels of cyclin E1 and E2 were detected in both TAM-sensitive (defined as TAM-S) and TAM-resistant (defined as TAM-R) [24] MCF-7 cells (Figure 2A). We found that CCNE2 expression levels were detected at higher levels in the TAM-R cells. In contrast, there were no significant differences in the expression levels of cyclin E1 between TAM-S and TAM–R cells (Figure 2A). Thus, these data support the previous papers that found higher levels of CCNE2 protein to be a predictor of poor outcome in breast cancer patients, especially in the context of TAM resistance [27].

We then tested whether targeting CCNE2 expression with food components could reverse TAM resistance in breast cancer patients. As described above (Figure 1A), the CCNE2 protein expression levels were detected at relatively higher levels in MDA MB-231 cells (ER-/PR-/Her2-) compared with MCF-7 cells (ER+/PR+/Her2-). Both cell lines were selected for screening different components isolated from natural products (data not shown). Interestingly, we found that the CCNE2 expression levels in MCF-7 cells were inhibited by quercetin (> 1 μM) in a

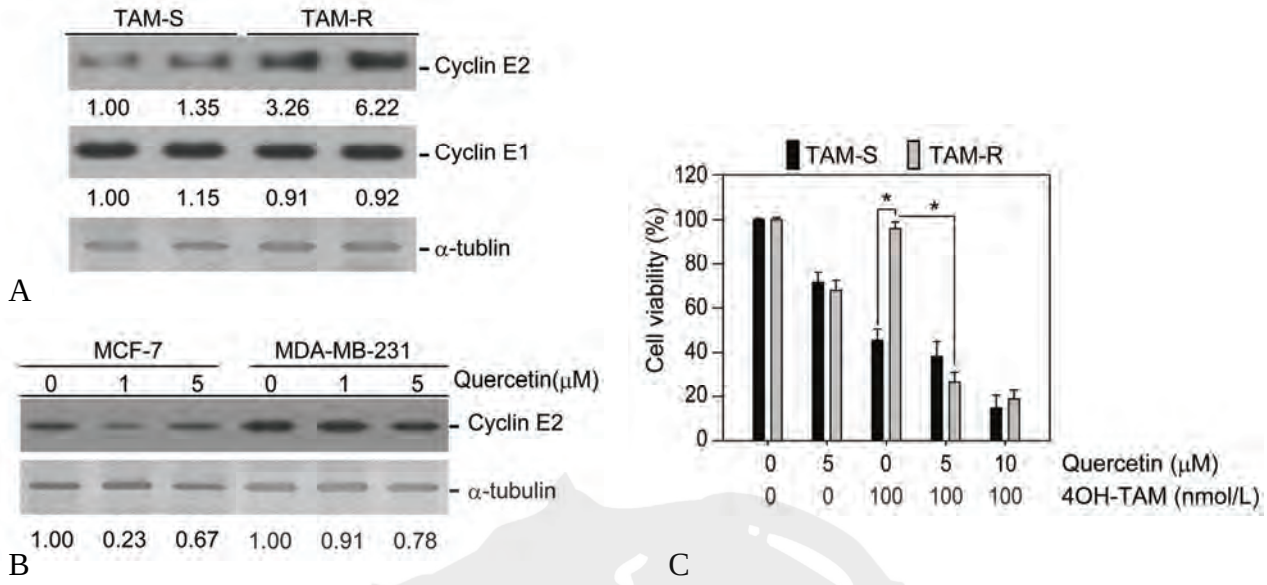

Figure 2. CCNE2 protein expression in human breast cancer cells was inhibited by quercetin. (A) The CCNE1 and CCNE2 protein expression levels were detected in TAM-S and TAM-R cells using immunoblotting analyses. (B) Human breast cancer cells (MCF-7 and MDA-MB-231) were treated with 1-5 μM quercetin for 24 hrs, and the CCNE2 protein expression level was detected using immunoblotting analyses. The α-tubulin protein expression levels were detected as loading controls. (C) Human breast cancer (MCF-7) and TAM-R cells were treated with quercetin, 4-OH-TAM, or a combination treatment of quercetin (5 μM) and 4-OH-TAM (100 nM) for 24 hrs, and an MTT assay was performed. The data in (C) represent the mean and 95% confidence interval (CI) of at least three experiments. The significance of the differences in cell viability between groups was analyzed using a Kruskal-Wallis (nonparametric) test, and each pairwise comparison was performed using a Mann-Whitney test. *A P-value of <0.05 was considered significant, and all statistical tests were two-sided

dose-dependent manner after 24 hrs. In contrast, this effect was not detected in MDA MB-231 cells (Figure 2B). A cell viability assay further demonstrated that TAM-R cells, which express relatively higher CCNE2 levels, were resistant to 4-OH-TAM (100 nM) treatment compared with TAM-S cells (Figure 2C, right; bar 5 vs. 6, $*P < 0.05$). However, a combination treatment of 4-OH-TAM (100 nM) and quercetin (5 μM) significantly sensitized the TAM-R cells to 4-OH-TAM (Figure 2C, right; $*P < 0.05$).

As described above, CCNE2 expression levels were associated with ER+/PR+/Her2- tumor patients (Table 2). CCNE2 upregulation following estrogen-mediated ER induction leads to an increase in the CCNE2/Cdk2 complex, which then triggers cell cycle progression from G1 into S phase [16]. To test whether the CCNE2 protein is involved in the estrogen-induced S-phase cell cycle progression, MCF-7 cells were treated with a pure estrogen antagonist (ICI 182780) [29] for 48 hrs to induce quiescence (Figure 3A). Then, the arrested MCF-7 cells were rescued with 100 nM estrogen, and flow cytometric analysis was performed. In this model, the entire cell population initiates cell cycle progression following estrogen treatment, reaching the S phase between 12 and 16 hrs later, the G2 phase at ~24-28 hrs, and dividing after 32 hrs [16] (data not shown). The CCNE2 protein levels were up-regulated prominently in both estrogen- treated

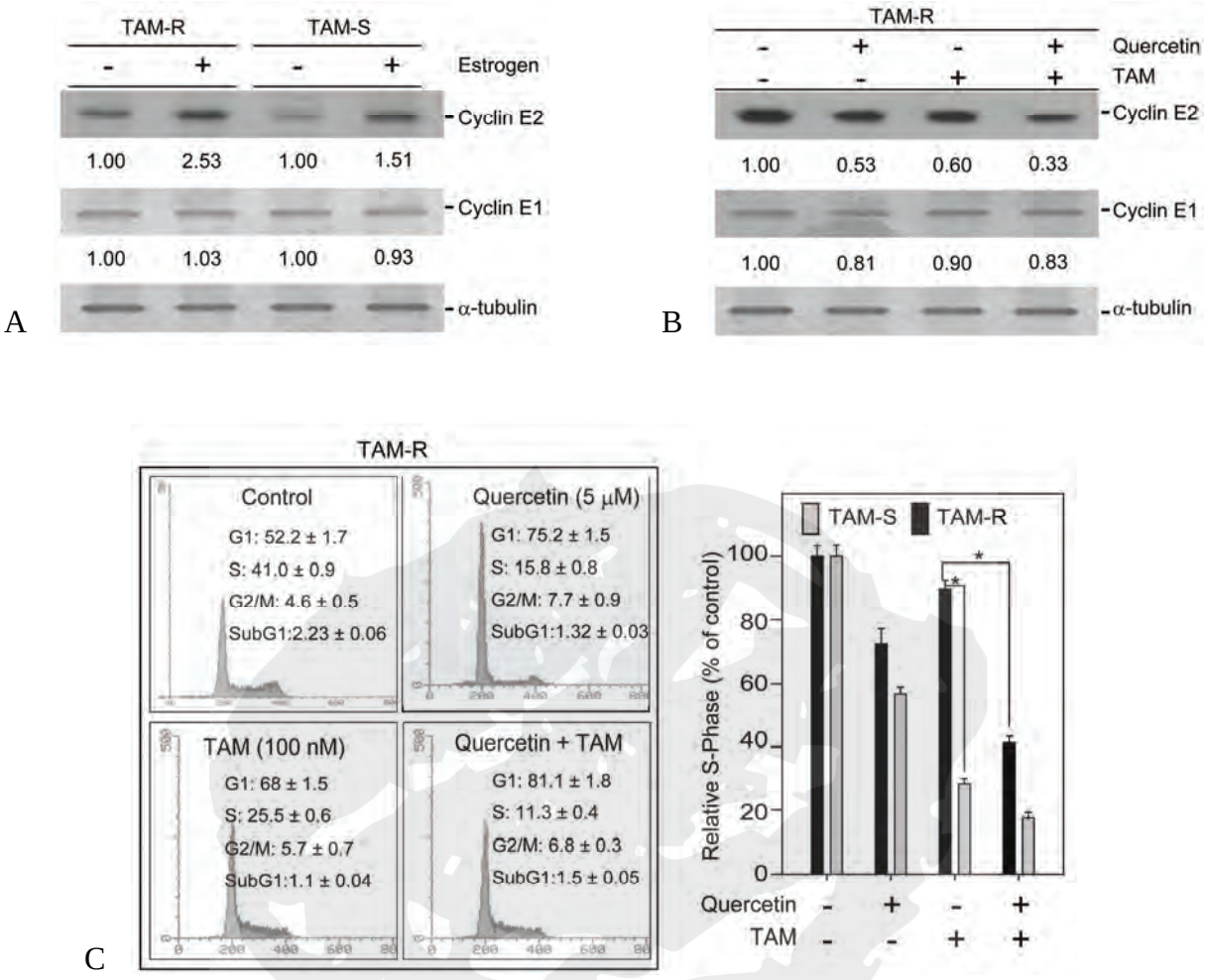

Figure 3. CCNE2 upregulation was detected in both TAM-S and TAM-R Mcf-7 cells. (A) Human breast cancer cells (MCF-7) were arrested for 48 hrs with 10 nM ICI182780 and then treated with 100 nM 17β-estradiol (estrogen) or vehicle (EtOH), and the cells were harvested over a course of 32 hrs for cell cycle analysis using flow cytometry. After 48 hrs of ICI 182780-induced cell quiescence, breast cancer cells (TAM-S, TAM-R) were treated with estrogen or EtOH for an additional 16 hrs, and the CCNE1 and CCNE2 protein expression levels were detected using immunoblotting analyses. The α-tubulin protein was detected as a loading control. (B) The TAM-R cells were treated with quercetin (5 μM), 4-OH-TAM (100 nM) or the combination for 16 hrs. Then, the cell lysates were collected, and CCNE1 and CCNE2 protein expression levels were detected using immunoblotting analyses. (C) The TAM-R cells were treated with quercetin (5 μM), 4-OH-TAM (100 nM) or the combination for 16 hrs. The cells were then harvested for cell cycle analysis using flow cytometry. In the left panel, the data are representative of at least three experiments, and the graphs represent the pooled results of analyses from at least three experiments. The data in the right panel represent the mean and 95% confidence interval (CI) of at least three experiments. The significance of differences in the relative S phase (% of control) between groups was analyzed using a Kruskal-Wallis (nonparametric) test, and each pairwise comparison was performed using a Mann-Whitney test. *A P-value of <0.05 was considered significant, and all statistical tests are two-sided

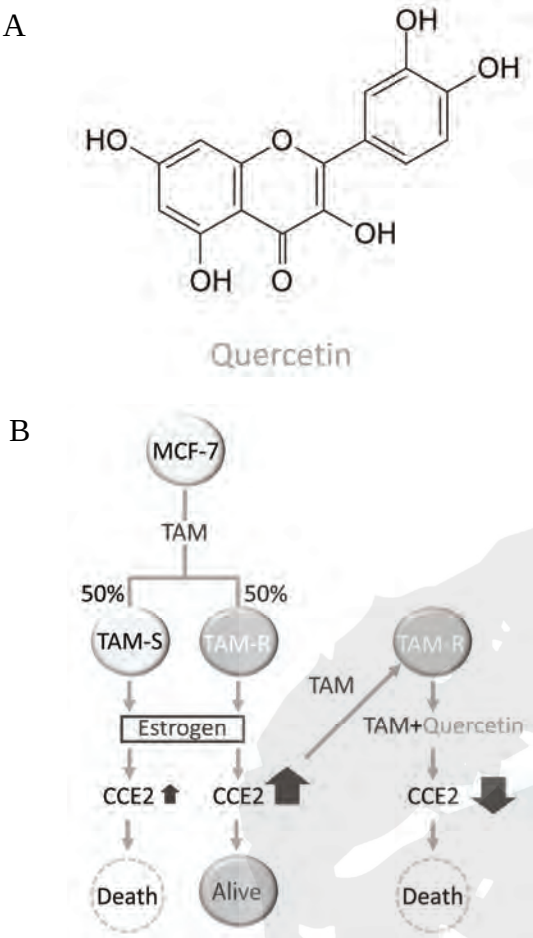

Figure 4. A schematic diagram is shown of quercetin-induced CCNE2 inhibition in TAM-R cells. (A) The chemical structure of quercetin is shown. (B) The luminal type a breast cancer patients were treated with TAM as a first-line therapy agent. Unfortunately, up to 50% of patients with metastatic disease do not respond to TAM. In this study, the MCF-7 (TAM-S and TAM-R) cells were treated with estrogen, and CCNE2 was induced. The estrogen-induced CCNE2 expression was inhibited in both cells. The combination treatment of TAM and quercetin significantly sensitized the TAM-R cells to TAM treatment by inhibiting CCNE2 expression. This study suggests that targeting CCNE2 expression with food components such as quercetin may overcome TAM resistance in breast cancer patients

TAM-S and TAM-R MCF-7 cells (100 nM, for 16 hrs; Figure 3A). In contrast, the cyclin E1 protein levels did not change significantly in either cell line (Figure 3A). To test whether the CCNE2 protein expression levels could be down-regulated by quercetin in the presence of TAM treatment in human breast cancer cells, the TAM-R MCF-7 cells were harvested 16 hrs after release from ICI 182780-induced quiescence (while in the S phase). The basal levels of the cyclin E1 and E2 proteins were detected using immunoblotting analyses (Figure 3B). We then treated with TAM (100 nM) in the presence or absence of quercetin (5 μM) 16 hrs (S-phase) after TAM-R cells were released from quiescence, and CCNE1 and CCNE2 protein expression levels were evaluated using immunoblotting analyses (Figure 3B). The results showed that unlike cyclin E1, the CCNE2 protein expression levels were inhibited in the quercetin-treated TAM-R cells in the presence or absence of TAM (Figure 3B). The CCNE2 protein expression levels were not affected by TAM treatment (Figure 3B).

The CCNE2/Cdk2 complex is required for S-phase cell cycle progression. Therefore, we hypothesized that CCNE2 inhibition in TAM-R cells by quercetin inhibits S phase cell cycle progression. We then tested whether quercetin could sensitize TAM-R cells to TAM. The TAM-R cells were treated with quercetin (5 μM), TAM (100 nM), or the combination, and the cell cycle distribution was then evaluated (Figure 3C). The results showed that quercetin significantly sensitized TAM-R cells to TAM via the inhibition of cancer cell entry to S phase from G1 phase (Figure 3C, $*P < 0.05$). These mechanisms help to provide a better understanding of the results shown in Figure 2C, which demonstrated that quercetin increased the TAM-induced cytotoxic effects on the TAM-R cells.

DISCUSSION

In this study, we identified the relationship between the overexpression of CCNE2 and TAM resistance, but not CCNE1 in breast cancer patients.

These results are similar to those results found in earlier reports indicating that CCNE1 is generally more strongly associated with poor patient outcome in ER- breast cancer patients, whereas CCNE2 is more strongly associated with poor outcome in ER+ breast cancer patients [13,14]. Another study demonstrated that higher CCNE2 expression is associated with a shorter metastasis-free survival following endocrine therapy [27]. Mechanistic studies have indicated that only cyclin E1 leads to the upregulation of both cyclin D1 and p21/Waf1/Cip1, and although the activity of the CCNE2/CDK2 complex is inhibited by p27/Kip1 [17], the truncated forms of cyclin E1 that confer resistance to anti-estrogen therapy are resistant to p21/Waf1/Cip1 and p27/Kip1 [30].

To determine whether the CCNE2 protein plays a critical role in the luminal type A (ER+/PR+/Her-2-) breast cancer cells, our study demonstrated that overexpression of CCNE2 protein was induced by estrogen, which then induces increased cell proliferation. Reduced estrogen-induced CCNE2 protein expression by ICI182780, an ER antagonist, inhibited cell cycle progression into the S phase. Such results indicate that estrogen was involved in the CCNE2-induced cell proliferation through activation of ER signaling pathways [16,27]. However, we demonstrated that CCNE2 protein expression levels were higher in TAM-resistant breast cancer cells. These results indicate that CCNE2, but not cyclin E1, may play an important role in resistance to anti-estrogen therapy. Accordingly, targeting CCNE2 expression may be a useful strategy for breast cancer therapy in human TAM-R cells. In this study, different compounds isolated from natural products were screened for inhibition of CCNE2 expression in human breast cancer cells (data not shown). Moreover, we found that CCNE2 inhibition induced by quercetin sensitized TAM-induced cytotoxicity in the TAM-R MCF-7 cells through blockage of the cell cycle entry to the S phase.

Herbs and other botanicals agents have served as medicines in every culture throughout human history, for good reason. Although scientists may be concerned about the unsafe use of botanical agents as cancer treatments, these agents contain biologically active constituents that have been identified as therapeutic agents. Many previous studies postulated that quercetin has the potential to be used as a chemosensitizer when used in combination treatment with anti-tumor agents (Table 1). In this study, the results indicated that a lower concentration of quercetin (> 5 μM) effectively reversed TAM resistance in TAM-treated breast cancer cells. Our other study also demonstrated that the combined treatment of both quercetin and luteolin (0.5 μM, each) has a significant effect on the inhibition of nicotinic receptor in human breast cancer cells, which then confers its anti-tumor properties on the formation of smoking-induced breast cancer [7]. These several studies have demonstrated that quercetin has been well-researched in conjunction with a combination of anti-tumor agents, tea components, and natural compounds that are known to have significant effects on the inhibition of different cancer cells growth, anti-angiogenesis, apoptosis, and anti-metastasis (Table 1).

On the other hand, studies have demonstrated that quercetin alone exerts its anti-tumor activity through different mechanisms (Table 1). For example, quercetin suppresses the epithelial-mesenchymal transition [31,32]. Quercetin inhibits proliferation in the ER-positive human breast cancer MCF-7 cells through reduction of nuclear factor erythroid 2-related factor 2 (Nrf2) signaling [33]. These data indicate that quercetin exerts anti-tumor effects in both ER-positive and ER-negative cells. These studies further indicate that the differences in quercetin-induced cell cycle inhibition in both ER-positive and ER-negative cells were concentration-dependent effects. Many previous articles have also indicated that quercetin can inhibit the environmental estrogen-induced proliferation of human breast carcinoma MCF-7 cells [34,35]. These results indicate that CCNE2 protein expression levels may play a central role in cell cycle progression in

cells arrested by quercetin. All of these data suggested that quercetin has clinical significance for application with anti-tumor therapy either as a chemopreventive or as a chemosensitizer agent.

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