EGFR-mediated G1/S transition contributes to the multidrug resistance in breast cancer cells

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Abstract Despite the improvement of strategies against cancer therapy, the multidrug resistance (MDR)is the critical problem for successful cancer therapy. Recurrent cancers after initial treatment with chemotherapy are generally refractory to second treatments with these anticancer therapies. Therefore, it is necessary to elucidate the therapy-resistant mechanism for development of effective therapeutic modalities against tumors. Here we demonstrate a phase-specific chemotherapy resistance due to epidermal growth factor receptor (EGFR) in human breast cancer cells. Thymidine-induced G1-arrested cultures showed upregulated chemosensitivity, whereas S-phase arrested cells were more resistant to chemotherapeutic agents. Overexpression of EGFR promoted the MDR phenotypes in breast cancer cells via accelerating the G1/S phase transition, whereas depletion of EGFR exerted the opposite effects. Furthermore, CyclinD1, a protein related to cell cycle, was demonstrated to be involved in above EGFR-mediated effects since EGFR increased the expression of CyclinD1, and the specific RNA interference against CyclinD1 could primarily abolish the EGFR-induced MDR phenotypes. These data provide new insights into the mode by which MDR breast cancers evade cytoxic attacks from chemotherapeutic agents and also suggest a role for EGFR-CyclinD1 axis in this process.

Keywords Multidrug resistance · EGFR · Cell cycle · CyclinD1 · Breast cancer

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Introduction

Malignant tumors can become resistant to conventional antineoplastic agents, which has been a major clinical problem in cancer treatment in general.Multidrug resistance (MDR) is mediated by complex mechanisms which are severe challenge for both basic and clinical researchers. The most investigated mechanisms with known clinical significance are: (a) activation of the cell membrane pumps effluxing different chemical substances from the cells; (b) activation of the enzymes of the glutathione detoxification system; (c) alterations of the genes and the proteins involved into the control of apoptosis [1]. However, the precise mechanisms of MDR have not yet been completely elucidated, suggesting the possible existence of unknown molecules and mechanisms responsible for the development of MDR.

The development of drug resistance phenotype could be accompanied by changes in morphological structure, proliferative potential and adhesion properties of cells as well as the changes in expression of proteins involved in cell cycle control [2–5]. It is becoming increasingly apparent that the cell cycle plays a critical role in regulating chemosensitivity of cancer cells. Cell cycle-mediated drug resistance is best described as a relative insensitivity to a chemotherapeutic agent because of the position of the cells in the cell cycle. On one hand, this is prevalent in

combination chemotherapy, where one chemotherapeutic agent can impact the cell cycle such that the next chemotherapeutic agent given immediately in sequence becomes less effective. The best example includes those combinations that involve taxanes [6, 7]. In view of the fact that taxanes is predominately an M-phase-specific drug, other agents that arrest tumor cells in G1 before they enter M phase would lead to a significant reduction in paclitaxel sensitivity. On the other hand, upon treatment of a cytotoxic agent, tumor cells that undergo cell cycle arrest may be protected from apoptosis and may ultimately acquisite the multidrug resistance phenotypes. For example, after induced by high-concentration, short-duration drug treatment of cisplatin, the proportion of SK-Hep-1/CDDP cells (the established multidrug-resistant hepatoma cell line) in the G2/M and S phases increased significantly [8]. Previous studies have also suggested that the most prominent effect of temozolomide (TMZ) in gliomas is G2/M arrest but not apoptosis, which results in the creation of cells with more aggressive phenotypes including drug resistance [9]. Thus, studies on mechanisms of cell cycle-mediated resistance are of paramount importance for further understanding of fundamental processes in formation of drug resistance phenotype in tumors, with the aim of searching the ways for overcoming such resistance.

In this study, we demonstrated the differential chemosensitivity of the G1 and S-synchronized breast cancer cells, and suggested a critical role for EGFR in impairing resistance in the S population. In addition, EGFR appears to exert its phase-specific antiapoptotic effect via consequent transcriptional activation of CyclinD1 to regulate the G1/S phase transition. These studies suggest EGFR-CyclinD1 axis as a promising therapeutic target against MDR in breast carcinomas.

Materials and methods

Cell culture

The human breast carcinoma cell line MCF7 and its multidrug-resistant counterpart MCF-7/Adr were purchased from ATCC and cultured under conditions provided by the manufacturer. Multidrug resistant sublines of MCF7 were obtained by culturing the cells in gradually increasing doses of Adriamycin (ADM) as described previously [10]. Cells which grew in 1, 3, 5 and 10 µM ADM were obtained after 2, 4, 7 and 11 months of culture with adriamycin, respectively. The stability of the resistant phenotype was determined by culturing continuously in medium with corresponding concentrations of ADM and assessing relative resistance after various periods of time up to 5 months.

Reverse transcription and quantitative real-time polymerase chain reaction

Quantitative RT-PCR was carried out using the basic procedure described previously [11]. The following are the primers used for the specific amplification of *CyclinD1*: forward: 5′-ccgtccatgcggaagatc-3′, reverse: 5′-atggccagcg ggaagac-3′; and *CDK4:* forward: 5′-agccgagcgtaagatc ccct-3′, reverse: 5′-cagctgctcctccattagga-3′.

Immunoblotting

Total protein was extracted from cells using RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA). 50 µg of protein extract/lane were electrophoresed, transferred to PVDF membranes, and incubated overnight with antibodies against EGFR (Santa Cruz Biotechnology Inc., Santa Cruz, CA), P-gp (Chemicon International, Temecula, CA), MRP1 (Santa Cruz) and ABCG2 (Santa Cruz), CyclinD1 (Cell Signaling Technology) and CDK4 (Cell Signaling Technology) respectively. Membranes were treated with the appropriate HRP-conjugated secondary antibodies (Invitrogen, Carlsbad, CA). Detection was performed using the reagents provided in the ECL+Plus kit (GE healthcare, Wauwatosa, WI).

Inhibition of EGFR and CyclinD1 expression by RNA interference

2×10^5 cells were seeded in 6-well plates in triplicates and after an overnight incubation, the cells were transfected with various concentrations of siRNA using HiPerfect Reagent (Qiagen) as suggested by the manufacturer's instructions. The small interference RNA used to target EGFR and CyclinD1 mRNA sequence were synthesized by Qiagen.

Luciferase reporter assay

Luciferase reporter plasmids pGL3-*CCND1*, pGL3-*CCND2*, and pGL3-*CCND3*, containing CyclinD1, CyclinD2, and CyclinD3 promoters, were generously provided by Peter G. Milner, CV Therapeutics, Palo Alto, California. Cells of 50% confluence in 24-well plates were transfected using Lipofectamine2000 (Invitrogen) and the luciferase activity was measured as described previously [12].

Cell proliferation assay

Cell proliferation in vitro was analyzed with MTT (Sigma, St. Louis, MO, USA). Briefly, 2×10^3 cells of each group

were plated per well in three 96-well microplates in 200 μl of medium. After 24, 48, and 72 h of culture respectively, 20 μl of MTT substrate (5 mg/ml in PBS) was added to each well, and the plates were returned to standard tissue incubator conditions for an additional 6 h. The medium was then removed, the cells were solubilized in 150 μl of dimethyl sulfoxide, and colorimetric analysis was performed (wavelength, 490 nm).

In vitro drug sensitivity assay

Cells were plated in 96-well plates at a density of 10^4 cells per well and further incubated for 24 h. The medium was then removed and replaced with fresh medium containing paclitaxel (Sigma), vincristine (Sigma), 5-fluorouracil (Alexis Biochemicals) and adriamycin respectively with varying PPC (plasma peak concentration, 0.1, 1 and 10 PPC) for another 48 h. After that, cells were stained with 20 μl sterile MTT dye (5 mg/ml; Sigma) at 37°C for 4 h followed by removing culture medium and mixing 150 μl of DMSO thoroughly for 10 min. Spectrometric absorbance at 490 nm was measured with a microplate reader. Each group contained three wells and was repeated for three times. The IC_{50} value was determined by the dose of drug that causes 50% cell viability.

In vivo drug sensitivity assay

The subrenal capsule assay was preformed to evaluate the sensitivity of different cell lines (MCF7/con, MCF7/EGFR and MCF7/EGFR/shRNACyclinD1) to ADM as described previously [12].

Statistical analysis

Statistics were calculated by SPSS software. The results are presented as mean ± standard errors (SEM). ANOVA, Student's t test analysis and Dunnett's multiple comparison tests were used to compare mean values. A P value of less than 0.05 was defined as statistical significance.

Results

Analysis of the cell cycle distribution in MDR breast cancer cells and their parental cells

The abnormal regulation of cell cycle is known as one of the characteristic features of the malignant cells. Here, we compared the cell cycle distribution in MCF7 and MCF/Adr cells, which showed different patterns of cell cycle arrest. More that 60% of MCF7/Adr cells were arrested at S phase, whereas most MCF7 cells were in G1 phase under normal culture conditions (Fig. 1a). We also evaluated the cell cycle progression patterns in the MDR variants of MCF7 cells cultured continuously in gradually increasing doses of ADM up to 10 μM. When compared to its parental line, induction of the MDR phenotype accompanied with the significant decrease of G1 phase percentage (from 76.3 to 20.1%) with S-phase arrest (from 11.8 to 73.4%). Moreover, we found that the level of S-phase arrest was closely associated with the degree of ADM resistance (Fig. 1b). These results indicate that changes in the cell cycle distribution correlate with acquisition of the MDR phenotypes in breast cancer cells.

The MDR phenotype is cell cycle-dependent in breast cancer cells

To determine if acquisition of the MDR phenotype was attributed to the S-phase arrest, MCF7 cells were synchronized with thymidine in the G1 phase or in the S phase after subsequent releasing. It was observed that with 6 h of thymidine treatment, IC_{50} value for adriamycin on the S-arrested cells was 8.5772 ± 2.035 μg/ml. The S-arrested cells were significantly more resistant to adriamycin in comparison with the cells arrested at G1 phase (2.0737 ± 0.392 μg/ml) or the unsynchronized controls (2.4842 ± 0.752 μg/ml). We also compared the cross-resistance to other anticancer drugs between the cells arrested at G1 and S phase respectively. The S-arrested cells had cross-resistance to Toxel, VCR and 5-FU, which was not observed in either the G1-arrested cells or the unsynchronized controls (Table 1).

Fig. 1 Correlation of S-phase arrest with the MDR phenotype in breast cancer cells. The cell cycle distribution of MCF7, MCF7/Adr cells (**a**) or MDR sublines of MCF7 cells (**b**) were assessed by flow cytometry
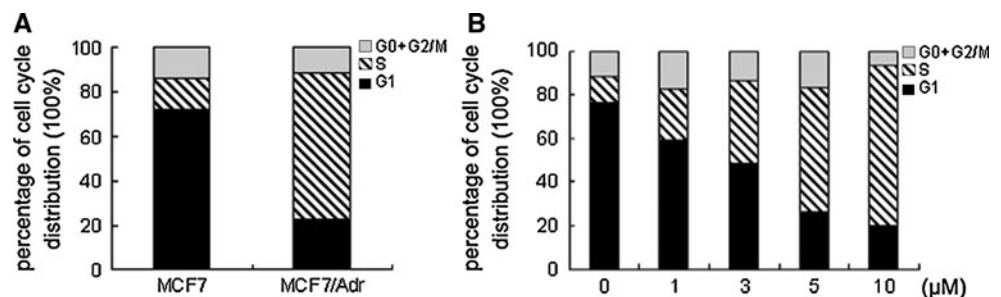
**Table 1** IC50 of chemotherapeutic agents in different cell cycle phases of MCF7 cells

Drugs	Unsynchronized	S	G1
ADR	2.6152 ± 1.1086	$8.5772 \pm 2.0353^*$	2.4842 ± 0.752
Toxel	3.5934 ± 1.1031	$7.4993 \pm 1.1682^*$	3.0494 ± 0.589

ADR Adriamycin, *Toxel* paclitaxel

IC50 values were expressed in μg/ml and were evaluated as reported in "Materials and methods" section

Standard deviations for all of the experiments performed in triplicate were less than 5%

* $P < 0.05$ versus control cells
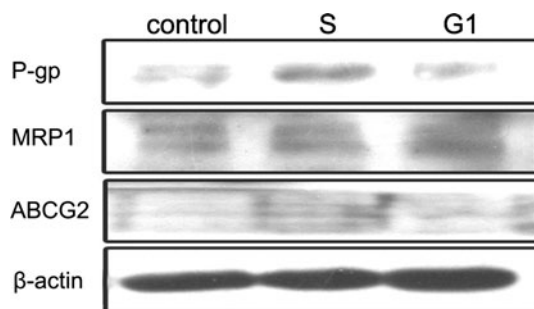
Fig. 2 Upregulation of P-gp and ABCG2 is cell cycle-dependent in breast cancer cells. After MCF7 cells were synchronized at G1 or S phase, the expression levels of P-gp, ABCG2 and MRP1 were determined by western blot analysis

To study the possible molecular mechanisms involved in the cell cycle-dependent MDR of breast cancers, the expression levels of 3 classical MDR molecules, P-glycoprotein (P-gp), multidrug resistant protein 1 (MRP1) and ATP-binding cassette superfamily G member 2 (ABCG2), were examined in the cells synchronized at different phases. The expressions of both P-gp and ABCG2 in the G1-arrested cells were remarkably higher than those in the S-arrested cells and the unsynchronized cells, indicating that P-gp and ABCG2 might mediate the cell cycle-dependent MDR of breast cancer cells (Fig. 2). However, the expression of MRP1 remained unchanged upon cell cycle synchronization (Fig. 2).

EGFR promotes G1/S transition in the cell cycle

EGFR, overexpressed in multidrug resistance tumor cells, plays a critical role in cell cycle progression. We examined the cell cycle distribution of the EGFR-transfected cells by flow cytometry. As shown in Fig. 3a, b, ectopic EGFR expression led to S-phase arrest in MCF7 cells, accompanied with enhanced tumor cell growth (Fig. 3c). In contrast, depletion of EGFR in MCF7/Adr cells overrode S-phase arrest and resulted in decreased tumor cell growth.

We further analyzed the effect of EGFR on cell cycles by synchronization. MCF7/EGFR cells were arrested at G1 phase by thymidine treatment and began entering into S phase at 4 h after releasing. The average releasing rate of cells from G1 to S phase in MCF7/EGFR was 45.7%, which was significantly higher than those of vector control cells (13.6%). Twelve hours later after releasing, most cells had gone into S phase. No significant difference was found in cell cycle profile between EGFR-transfected cells and control cells at later times (Fig. 3d). Taken together, these data strongly indicate that EGFR plays an important role in accelerating G1 to S phase transition in the cell cycle, and might thus promoting the MDR phenotype of breast cancer cells.

Cyclin D1 is involved in G1/S transition of breast cancer cells regulated by EGFR

To identify molecules that are regulated by EGFR and are responsible for the effects caused by EGFR in breast cancer cells, gene array was used to screen for target molecules. Together, 373 genes were found to be up-regulated in EGFR-transfected cells and 92 genes were down-regulated. Among them, 62 genes related to cell progression, especially CyclinD1, which is a key molecular in G1/S phase transition regulation, was found to be upregulated in the EGFR-transfected cells. Therefore, we examined the expression of CyclinD1 in breast cancer cells after EGFR transfection. Increased expression of CyclinD1 and CDK4 was confirmed by qRT-PCR and western blotting in the EGFR-transfected cells. In contrast, depletion of EGFR was found to suppress expression of CyclinD1 and CDK4 in MCF7/Adr cells (Fig. 4a, b).

To investigate the possible mechanisms involved in the regulation of CyclinD by EGFR, we performed dual-luciferase-reporter assay. Luciferase reporter plasmids pGL3-*CCND1*, pGL3-*CCND2*, and pGL3-*CCND3*, containing CyclinD1, CyclinD2, and CyclinD3 promoters, respectively, were transiently transfected into MCF7/Adr and MCF7/Adr/EGFRRNAi cells together with the pRL-TK. As shown in Fig. 4c, the intensity of luciferase luminescence in MCF7/Adr cells cotransfected with either pGL3-*CCND1*, pGL3-*CCND2*, or pGL3-*CCND3* was 6.82-, 2.57-, and 3.44-fold higher than that of the control cells, respectively, indicating that EGFR triggered transactivation of CyclinD. However, deletion of EGFR inhibited the transactivation of CyclinD in MCF7/Adr cells, which overexpress EGFR (Fig. 4c).

Effects of EGFR on cell cycle-related multidrug resistance in breast cancer cells

Since our previous results demonstrated that the MDR phenotype in breast cancer cells is cell cycle-dependent, we

Fig. 3 Effects of EGFR on the cell cycle distribution in breast cancer cells. The expression level of EGFR (a), the cell cycle distribution (b) and the proliferative rate (c) of MCF7/EGFR and MCF7/Adr/EGFRRNAi transfectants were determined by immunoblotting, flow cytometry and MTT assay, respectively. d MCF7/EGFR cells were arrested at G1/S boundary by adding thymidine (2 mM) at 0 h. Four hours after releasing, the cells began entering into S phase. The average releasing rate of cells from G1 to S phase in MCF7/EGFR was significantly higher than those of vector control cells. There was no significant difference in cell cycle profile between EGFR-transfected cells and the control cells at later times. **$P < 0.05$ versus the controls cells
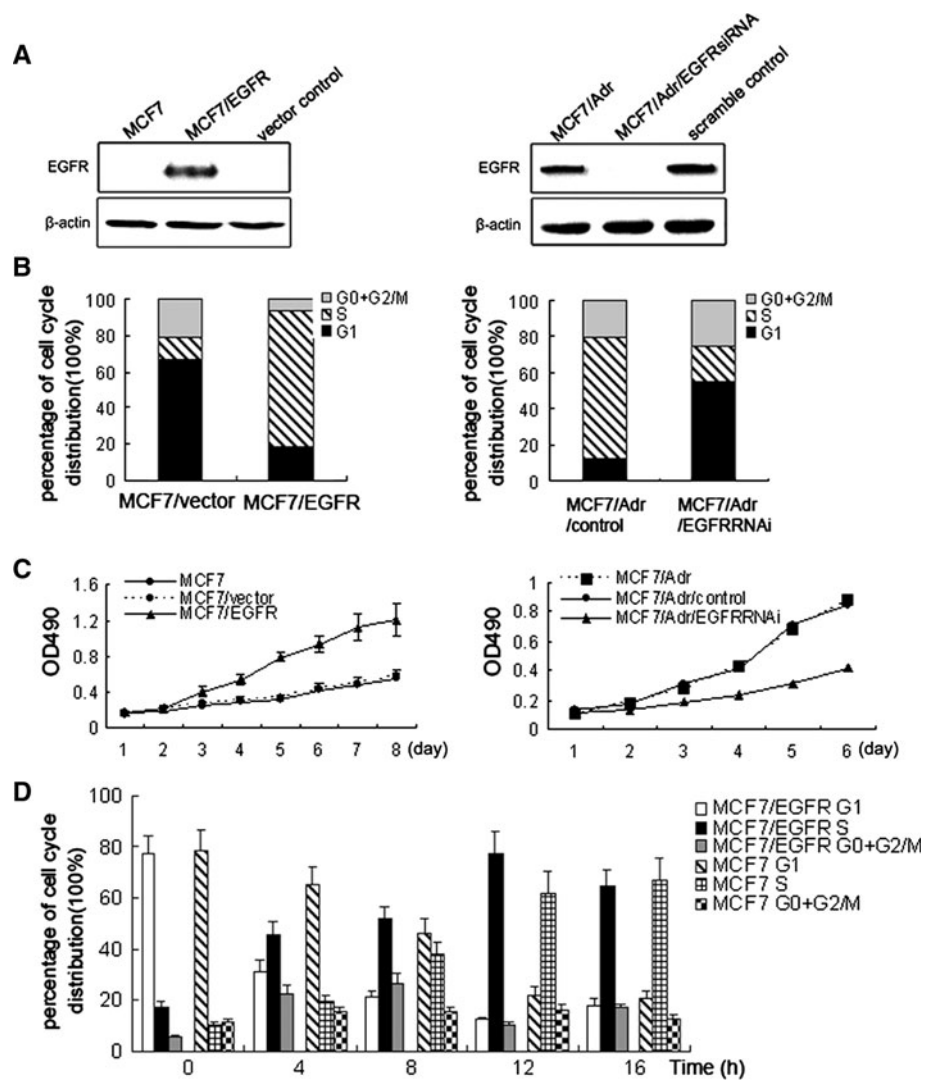

hypothesized that EGFR would effect the MDR phenotype via modulating cell cycle regulatory machinery. To test this hypothesis, in vitro effects of chemotherapeutic agents on the growth of MCF7/EGFR were evaluated by MTT assay. As shown in Table 2, EGFR had different effects on drug sensitivity, depending on the drug used. The MCF7/EGFR cells showed a >5-fold increased resistance to P-gp-related drugs ADM, Toxel, VCR and a >2.5-fold increased resistance to P-gp-nonrelated drugs 5-FU as compared to the control cells ($P < 0.05$). However, all these effects were remarkably reversed upon CyclinD1 depletion.

We also investigated whether EGFR is required for the cell cycle-related MDR phenotypes of breast cancer cells in vivo. As shown in Fig. 5, relatively inhibitory rates of ADM to MCF7/EGFR cells were lower than those to MCF7 control cells, suggesting that EGFR overexpression could exert drug resistance on MCF7 cells. We then determined in vivo reactivity of CyclinD1-related transfectants to adriamycin. Figure 5 exhibited a strong tumor growth inhibitory effect of adriamycin treatment in the MCF7/control and MCF7/EGFR-shRNACyclinD1 tumors ($P < 0.05$), suggesting that in vivo implant of MCF7/EGFR were more resistant to ADM as compared to that of MCF7/cont and MCF7/EGFR-shRNACyclinD1, respectively. These data suggested that EGFR-CyclinD1 axis might confer cell cycle-dependent MDR phenotypes on breast cancer cells.

Discussion

Cancer progression has been suggested to involve the loss of cell-cycle checkpoint controls that regulate the passage through the cell-cycle [13]. Although no difference in the cell cycle duration between tumor cells is found, the proportion of dividing cells in tumors with rapid growth is much higher than that in tumors with lower proliferative activity. Combined with the fact that tumors with different
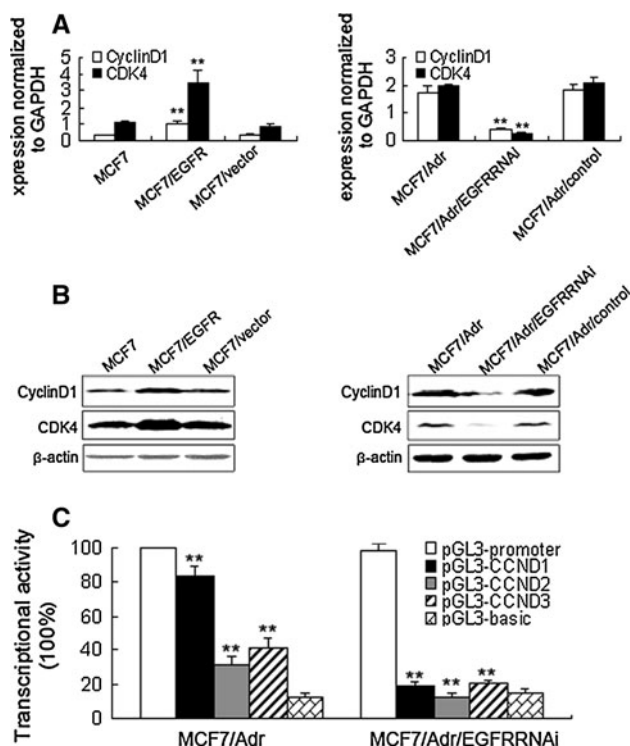
Fig. 4 The inducible effect of EGFR on CyclinD1. The mRNA (a) and protein expression (b) of CyclinD1 and CDK4 in MCF7/EGFR and MCF7/Adr/EGFRRNAi cells were assessed by qRT-PCR and western blot, respectively. c Relative luciferase activity of CyclinD promoters in MCF7/Adr cells transfected with or without EGFR siRNA were evaluated by dual luciferase reporter assay. **$P < 0.05$ versus the controls cells

Fig. 5 Modulation of MDR phenotype by EGFR-CDK4 axis in vivo. After in vivo ADM treatment for 8 days, the exact length and width of MCF7/EGFR and MCF7/EGFR/shCDK4 tumors were measured and the relative growth rate was determined. Bars represent the mean of triplicate samples. Data are representative of three independent experiments. **$P < 0.05$ versus the controls cells

growth kinetics usually have different sensitivity to antineoplastic agents, these raise the question of whether the differences in cytotoxicity are simply due to variations in the chemosensitivity of the cells in the different cell cycle phases. In the present study, we found that the cell cycle distribution of MCF7/Adr cell line exhibited a higher percentage of cells in S-phase in comparison with its parental cells, which accumulate mainly in G1 phase. The degree of S-phase arrest was positively associated with the levels of MDR and proliferative rate in breast cancer cells. It has been suggested that tumor with a more rapid growth rate, which is directly proportional to its percentage of S and G2/M phase cells in the DNA distribution, might be more sensitive to chemotherapy. However, we observed
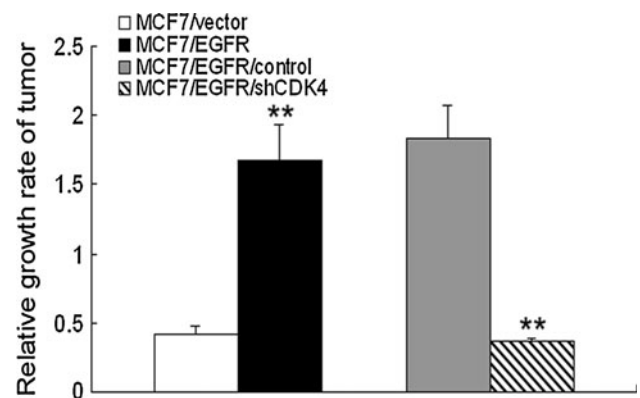
enhanced chemoresistance in breast cancer cells synchronized at S phase, whereas a dramatic increased sensitivity to chemotherapeutic agents was detected in the cells at G1 phase. These results implied that the most likely factor that determines the susceptibility of breast cancer cells to chemotherapeutic agents, and thus the tumor cell growth, lies in the cell cycle stages.

Epidermal growth factor receptor (EGFR), abundantly expressed in MDR tumor cells [14, 15], was found to promote G1 to S transition in the cell cycle of breast cancer cells. In mammalian cells, there are two classes of cyclin-dependent kinase (CDK) that function at the G1/S phase transition. G1 progression depends on the sustained expression of D-type cyclins, which, in turn, depends on continuous mitogenic stimulation and provides a link between mitogen signaling and the cell-cycle machinery [16, 17]. In our study, the expression of CyclinD1 and CDK4 was found to be upregulated in cells transfected with EGFR. Gene reporter assay suggested that EGFR could stimulate the promoter activity of CyclinD. Taken together, EGFR might facilitate cells cycle transition from G1 to S phase by accelerating the transcription of CyclinD1.

EGFR is a transmembrane glycoprotein that constitutes one of four members of the erbB family of tyrosine kinase

Table 2 IC50 of chemotherapeutic agents in EGFR transfectants

Drugs	MCF7/vector	MCF7/EGFR	MCF7/Adr	MCF7/Adr- EGFR siRNA
ADR	2.615228 ± 1.1085	4.262311 ± 0.4949*	17.54526 ± 3.1202	7.839379 ± 0.7596*
Toxel	3.593475 ± 1.1031	16.14693 ± 2.9152*	26.77999 ± 1.4857	7.627371 ± 1.6012*

IC50 values were expressed in μg/ml and were evaluated as reported in "Materials and methods" section

Standard deviations for all of the experiments performed in triplicate were less than 5%

* $P < 0.05$ versus control cells

receptors. Binding of EGFR to its cognate ligands leads to autophosphorylation of receptor tyrosine kinase and subsequent activation of signal transduction pathways that are involved in regulating cellular proliferation, differentiation, and survival. Although present in normal cells, EGFR is overexpressed in a variety of tumor cell lines and has been associated with poor prognosis and decreased survival [18]. However, the mechanisms related with this process are still not fully elucidated. Here, we demonstrated that overexpression of EGFR greatly promoted the MDR phenotype of breast cancer both in vitro and in vivo. These effects were primarily abolished by disrupting CyclinD1 expression, indicating that CyclinD1-mediated G1/S transition is responsible for acquisition of chemoresistance induced by EGFR. It has been acknowledged that EGFR overexpression correlates with increased resistance to various chemotherapies. For example, activation of EGFR promotes the development of drug resistance and cellular motility, effects that could be responsible for a more virulent behavior and poor prognosis of breast cancer [19]. Also, inhibition of EGFR by gefitinib is found to directly inhibit the function of P-gp in multidrug resistant cancer cells [20]. Our studies present a novel model in which CyclinD1 acts as one of the downstream effectors of EGFR and EGFR-CyclinD1 axis accounts for the cell cycle-related MDR phenotypes in breast cancer cells. Since a number of EGFR inhibitors have been developed that can restore chemosensitivity and arrest tumor growth, a better understanding of the molecular mechanisms of action of EGFR in this study may contribute to the development of further strategies for integration of EGFR inhibitors with chemotherapy or radiation to potentiate their anticancer activity.

Conflict of interest We have no conflict of interest.

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