Original Article

c-Src regulates cell cycle proteins expression through protein kinase B/glycogen synthase kinase 3 beta and extracellular signal-regulated kinases 1/2 pathways in MCF-7 cells

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We have demonstrated that c-Src suppression inhibited the epithelial to mesenchymal transition in human breast cancer cells. Here, we investigated the role of c-Src on the cell cycle progression using siRNAs and small molecule inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2). Western blot analysis demonstrated the down-regulation of cyclin D1 and cyclin E and up-regulation of p27 Kip1 after c-Src suppression by PP2. Incubation of cells in the presence of PP2 significantly blocked the phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2), protein kinase B (AKT), and glycogen synthase kinase 3 beta (GSK3β). Specific pharmacological inhibitors of MEK1/2/ERK1/2 and phosphatidylinositide 3-kinase/AKT pathways were used to demonstrate the relationship between the signal cascade and cell cycle proteins expression. The expression of cyclin D1 and cyclin E were decreased after inhibition of ERK1/2 or AKT activity, whereas the p27 Kip1 expression was increased. In addition, knockdown of c-Src by siRNAs reduced cell proliferation and phosphorylation of ERK1/2, AKT, and GSK3β. After c-Src depletion by siRNAs, we observed significant down-regulation of cyclin D1 and cyclin E, and up-regulation of p27 Kip1. These results suggest that c-Src suppression by PP2 or siRNAs may regulate the progression of cell cycle through AKT/GSK3β and ERK1/2 pathways.

Keywords breast cancer; cell cycle proteins; c-Src suppression; PP2; siRNAs

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Introduction

Breast cancer is now the most prevalent cancer in women worldwide [1]. Continuous chemotherapy inevitably results in drug resistance and cancer progression with uncontrolled growth and metastasis. One of the important mediators of the process is c-Src. c-Src is a 60-kDa non-receptor tyrosine kinase and its activation is significantly associated with tumor progression. A recent study has shown that c-Src could interact with focal adhesion kinase, and subsequently phosphorylate p130Cas and paxillin, regulate cell adhesion and migration [2]. Another study has shown that c-Src could phosphorylate tyrosine 845 of epidermal growth factor receptor (EGFR) that is required for mitogenic cellular survival signals [3, 4]. In addition, c-Src could also modulate the activity of phosphatidylinositide 3-kinases (PI3Ks), signal transducer and activator of transcription 3, and GTPase-activating proteins towards Rho-like small GTPases (RhoGAP) through phosphorylation of these targets [4, 5]. As a selective c-Src inhibitor, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) can inhibit the stimulation of angiogenesis by E-selectin [6] and reduce urokinase-type plasminogen activator receptor expression in colon cancer cell [7, 8].

Cell cycle progression is delicately regulated by positive regulators, such as cyclins and cyclin-dependent kinases (CDKs), and negative regulators, such as CDK inhibitors (CDKIs). Passage through G1 phase to S phase of cell cycle is promoted through assembly of cyclin D1 with CDK4 followed by cyclin E binding to CDK2 [9, 10]. Cyclin D1, known as G1 cyclin, is the limiting factor of G1–S transition during cell cycle progression [11, 12]. On the other hand, G1–S transition of cell cycle is inhibited by CDKIs, such as p21 WAF/Cip1, p27 Kip1, p57 Kip1, p15 INK4b, and p16 INK4a [9]. Among different CDKIs, p27 Kip1 is frequently down-regulated in various cancers including breast cancer [13].

We have reported that c-Src suppression markedly inhibited migration and epithelial to mesenchymal transition of breast cancer cells [14]. In this study, we observed that c-Src suppression may regulate the expression of cell cycle proteins in human breast cancer MCF-7 cells. The down-regulation of cyclin D1 and cyclin E and up-regulation of p27 Kip1 after c-Src suppression were demonstrated by...
western blot analysis. The inhibition of c-Src activity led to the decrease of extracellular signal-regulated kinases 1/2 (ERK1/2), protein kinase B (also known as AKT), and glycogen synthase kinase 3 beta (GSK3β) phosphorylation, which may involve in the regulation of cell cycle proteins expression. Using specific inhibitors of MEK1/2/ERK1/2 and PI3K/AKT pathways, we confirmed the relationship of these downstream pathways with cell cycle proteins expression. These results provide insights into the possible signaling pathway by which c-Src regulates cell cycle progression.

Materials and Methods

Reagents

The inhibitors, including PP2 (Merck, Whitehouse Station, USA), U0126 (Merck), and LY294002 (Merck), were dissolved in dimethyl sulfoxide (DMSO, Sigma, St Louis, USA). Polyclonal antibodies to phospho-AKT (S473), phospho-GSK3β (S21/9), GSK3β, and p27 Kip1 were obtained from Cell Signaling Technology (Cambridge, USA). Monoclonal antibodies to cyclin E, ERK1/2, phospho-ERK1/2 (T202/Y204), and AKT were obtained from Cell Signaling Technology. Monoclonal antibody to cyclin D1 was obtained from Santa Cruz Biotechnology (Santa Cruz, USA). Monoclonal antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from Proteintech Group (Chicago, USA). Horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, USA).

Cell culture

Human breast cancer MCF-7 cells were obtained from American Type Culture Collection (Manassas, USA). Cells were seeded at an initial concentration of 10^5 cells/ml in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, Pittsburgh, USA), 3.75 g/L sodium bicarbonate, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured at 37°C in a humidified incubator containing 5% CO2. MCF-7 cells were treated with 10 μM PP2 (Merck) for 12 h, 24 h, 30 h or transfected with siRNA-Src for 48 h as described before [14].

Cell growth assay

MCF-7 cells were seeded in 96-well plates (Corning, Steuben County, USA) with complete culture medium (DMEM containing 10% FBS) and grown for 24 h. Cells were then cultured with U0126, LY294002, or siRNA-Src for 48 h. At the end of the treatment period, 20 μl 3-(4,5)-dimethylthiazol-(2,5)-diphenyltetrazoliumromide (MTT; Amresco, Solon, USA) was added to each well, and cells were incubated at 37°C for 3 h. The reacting product formazan crystals were dissolved in 200 μl DMSO after removing the supernatant. After plates were shaken for 15 min, the optical density of the wells was measured at the wavelength of 570 nm with a Microplate Reader (Bio-Rad, Hercules, USA). Each concentration of inhibitors was performed in three wells, and cell growth assay was repeated for three times.

Cell cycle analysis

For cell cycle analysis, MCF-7 cells were grown to 80% confluence and then cultured with DMEM containing 0.2% FBS for 30 h to synchronize the cell cycle at G0/G1 phase as reported before [15, 16]. After different treatments, MCF-7 cells were harvested and washed with ice-cold staining medium (PBS, 5% FBS, 0.05% NaN3) and fixed in 70% cold ethanol at −20°C for 1 h. Cells were centrifuged and resuspended in 500 μl staining medium. Samples were then treated with RNase A (Sigma) and stained with 10 μg/ml propidium iodide (Sigma). DNA content and distribution of individual cells in different phases of the cell cycle were assessed by using an FACSCalibur flow cytometer (BD Biosciences, San Jose, USA) and analyzed with CellQuest software (version 7.5.3, BD Biosciences). The ranges for G0/G1, S, and G2/M phase cells were established based on their corresponding DNA content of histograms. At least 10,000 cells per sample were analyzed in the gate regions and used for calculations.

Western blot analysis

MCF-7 cells were cultured in the presence of inhibitors (10 μM PP2, 20 μM U0126 or 20 μM LY294002) or the same concentration of DMSO (as control). Cells were collected after inhibitor treatment or transfection with siRNA-Src for indicated time. For each sample, 20 μg of cell lysates were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto polyvinylidene difluoride membrane (Millipore, Billerica, USA) and incubated with primary antibodies at optimal dilution at 4°C overnight, followed by incubation with secondary antibody (Jackson ImmunoResearch) at 37°C for 1 h. The immunoblot was visualized with Enhanced Western Luminescent Detection Kit (Vigorous, Beijing, China).

Statistical analysis

All statistical analyses were performed using SigmaStat software (Version 3.5). Differences between different groups were assessed by one-way analysis of variance. P < 0.05 was considered to indicate statistical significance.

Results

The effect of c-Src inhibitor PP2 on cell cycle proteins in MCF-7 cells

Our previous studies have shown that c-Src activity is required for the proliferation of MCF-7 cells [17]. The
growth of MCF-7 cells can be inhibited in the presence of 10 μM PP2. Additionally, the phosphorylation of c-Src is greatly inhibited by 10 μM PP2 although the total level of c-Src is not changed [14]. To investigate whether c-Src suppression affects the expression of cell cycle proteins, we carried out western blot analysis to examine the alteration in protein levels. The expression of cyclin D1 and cyclin E was down-regulated after PP2 treatment, whereas the p27 Kip1 was significantly up-regulated [Fig. 1(A)]. In addition to slowing cell growth, we also observed that PP2 induced G1 arrest during cell cycle progression in MCF-7 cells. FACS analysis revealed that about 60.33% of MCF-7 cells were in G0/G1 phase in 10% FBS-supplemented DMEM. After PP2 treatment for a whole cell cycle, cells in G1 phase increased from about 60.33% (control) to 67.95% (12 h), 72.90% (24 h) and 77.48% (30 h). At the same time, cells in S and G2/M phases dramatically decreased [Fig. 1(B)].

c-Src mediated the phosphorylation of ERK1/2, AKT and GSK3β

As the indirect downstream targets of c-Src, ERK1/2 and AKT have been known to be involved in the regulation of cell proliferation in different cell types [18, 19]. We next examined the effect of c-Src inhibitor PP2 on ERK1/2 and AKT activation in MCF-7 cells. Phosphorylation of ERK1/2, AKT, and GSK3β was greatly inhibited by PP2 in a time-dependent manner. In contrast, the total levels of ERK1/2, AKT, and GSK3β did not change after PP2 treatment (Fig. 2).

ERK1/2 and AKT mediated the proliferation of MCF-7 cells

To assess whether ERK1/2 and/or AKT activation altered cell cycle proteins expression, we used the MEK1/2 (up-stream activator of ERK1/2) inhibitor U0126 and PI3K (up-stream activator of AKT) inhibitor LY294002. The effect of U0126 and LY294002 on cell proliferation was evaluated using MTT assay in MCF-7 cells. Treatment with U0126 or LY294002 alone induced a dose-dependent inhibition of MCF-7 cell proliferation [Fig. 3(A,B)]. In comparison, the inhibition effect of U0126 was weaker than that of LY294002. We chose 20 μM U0126 and 20 μM LY294002 in the subsequent experiments due to the significant inhibitory effect on cell proliferation at this concentration. U0126 inhibited ERK1/2 phosphorylation and LY294002 inhibited AKT phosphorylation [Fig. 3(C,D)], which supported the efficacy of the inhibitors. With consideration of inhibition of ERK1/2 and AKT activity by PP2, these data demonstrated...
that ERK1/2 and AKT pathways may be activated and regulated by c-Src during the proliferation of MCF-7 cells.

Role of ERK1/2 and AKT in cell cycle proteins expression

We next determined the role of ERK1/2 and AKT in regulation of cell cycle protein expression using the inhibitors U0126 and LY294002. As shown in Fig. 3(E,F), U0126 and LY294002 treatment decreased the expression of cyclin D1 and cyclin E, whereas p27 Kip1 expression was increased. These results suggested that cyclin D1, cyclin E, and p27 Kip1 expression may be mediated by AKT and ERK1/2 pathways.

Knockdown of c-Src by siRNAs induced reduction of cell proliferation, decrease of ERK1/2 and AKT phosphorylation, and alteration of G1-associated regulatory proteins

To confirm the role of c-Src suppression in regulation of cell cycle proteins expression, we used siRNA strategy to knockdown c-Src specifically. The effect of c-Src silencing has been reported previously [14]. MTT assay showed that,
c-Src knockdown induced about 20% decrease in MCF-7 cells proliferation [Fig. 4(A)]. We further examined the effect of c-Src depletion on the phosphorylation of ERK1/2, AKT, and GSK3β and the expression of cell cycle proteins. As shown in Fig. 4(B), c-Src knockdown reduced the phosphorylation of ERK1/2, AKT, and GSK3β. The significant down-regulation of cyclin D1 and cyclin E and up-regulation of p27 Kip1 were demonstrated by western blot analysis [Fig. 4(C)]. FACS analysis indicated that G1 arrest was also observed after c-Src suppression [Fig. 4(D)]. But the relative cell numbers in G1 phase increased less than that of PP2 treatment [Fig. 4(D)]. These results were consistent with the results obtained using c-Src inhibitor PP2, further supporting that the PI3K/AKT and ERK1/2 pathways may involve in alteration of cell cycle proteins after c-Src suppression in MCF-7 cells.

**Discussion**

There are currently several treatment protocols available for breast cancer in terms of chemotherapy, hormonal therapy, biological therapy, and radiation therapy [20–22]. Some drugs are still being evaluated including those targeting c-Src kinase [5, 23]. It is well-known that phosphorylation of...
c-Src could activate downstream signaling pathways to regulate many cellular processes [24]. In our earlier study using c-Src suppression by PP2 or siRNAs, we have shown the efficiency of c-Src suppression in inhibiting cell growth, migration, and epithelial to mesenchymal transition in human breast cancer cells [14, 17]. This study further explored the mechanism by which c-Src regulated cell cycle progression.

Cyclin D1 has been shown to be downstream of ERK1/2 and PI3K/AKT pathways [25, 26]. Recent studies suggested that transcriptional regulation of cyclin D1 requires sustained levels of active ERK1/2 [27]. Our results demonstrated that the inhibition of ERK1/2 activity by U0126 induced down-regulation of cyclin D1, indicating the role of ERK1/2 in the regulation of cyclin D1 expression. Additionally, GSK3β-mediated phosphorylation of cyclin D1 at T286 leads to proteosomal degradation of cyclin D1 [28–30]. Phosphorylation of GSK3β, a downstream target of PI3K/AKT pathway, was inhibited by c-Src inhibitor PP2 and siRNAs. Together, these results suggest that ERK1/2 and AKT/GSK3β pathways may contribute to c-Src-mediated stabilization of cyclin D1.

The reduced expression of p27 Kip1 is frequently observed in most human tumors and is correlated with tumor progression and poor patient survival, which is especially obvious in hormone-receptor-positive tumors [31, 32]. Here, we showed that c-Src suppression by PP2 or siRNAs induced significant up-regulation of p27 Kip1 in MCF-7 cells. A previous study has shown that most chemopreventive anticancer agents specifically up-regulate the expression of p27 Kip1 [33]. It is known that AKT can phosphorylate and repress the forkhead family of transcription factors that transactivate the p27 Kip1 promoter [34, 35]. In addition, AKT can directly phosphorylate p27 Kip1, resulting in the nuclear export of p27 Kip1 [36]. EGFR overexpression or Her2/ErbB2 amplification were frequently observed in human breast cancers [37]. Estrogen receptor (ER) is expressed in a large proportion of human breast cancers. Activated EGFR family receptor tyrosine kinases or liganded ER activate c-Src to promote cell cycle progression [3, 38]. Overexpression of EGFR, Her2 or estrogen-stimulated breast cancer proliferation increases p27 proteolysis [38, 39].

Our data showed that c-Src suppression down-regulated cyclin D1 and cyclin E and up-regulated p27 Kip1. The possible explanation is that c-Src inhibition increases p27 stability. This is consistent with the previous report that phosphorylation of p27 at tyrosine 74 and tyrosine 88 by c-Src impairs the Cdk2 inhibitory action of p27 and reduces its steady-state binding to cyclin E–Cdk2 to facilitate cyclin E–Cdk2-dependent p27 proteolysis. In tamoxifen-resistant breast cancer cell lines, c-Src inhibition can increase p27 levels and restore tamoxifen sensitivity. Tyrosine phosphorylation of p27Kip1 is involved in the activation of p27Kip1–cyclin D1–Cdk4 complexes and releases the inhibition of Cdk2 [40, 41]. Phosphorylation of p27Kip1 regulates assembly and activation of cyclin D1–Cdk4 [41], permitting phosphorylation and inactivation of Rb, synthesis of cyclin E, and progression into S-phase. Marcotte et al. [42] demonstrated that p27Kip1 tyrosine phosphorylation is highly dependent on c-Src in PyVmT tumor cells.

Cyclin E, which controls the transition from G1 to S phase, was down-regulated after c-Src suppression by PP2 or siRNAs. The turnover of cyclin E was reported to be modulated by assembly of cyclin E–CDK2 complexes and by two kinases, GSK3, and CDK2 [43]. Our results demonstrated that the inhibition of AKT/GSK3β pathway may contribute to the down-regulation of cyclin E after c-Src suppression.

In conclusion, we explored the mechanism by which c-Src regulated cell cycle progression in MCF-7 cells. c-Src suppression by PP2 or siRNA may mediate G1-associated cyclin D1, cyclin E, and p27 Kip1 through inhibition of AKT/GSK3β and ERK1/2 pathways. The pivotal role of c-Src in cell growth, migration, and cell cycle progression potentially urges the specific inhibitors for cancer treatment.

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References

c-Src regulates cell cycle progression


