Increase in CIP2A expression is associated with doxorubicin resistance

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The cancerous inhibitor of protein phosphatase 2A (CIP2A) increases the migration and metastasis of various cancer cells. Overexpression of CIP2A has been shown to increase the proliferation of MDA-MB-231 cells. We thus assessed whether CIP2A expression is associated with sensitivity to doxorubicin. MDA-MB-231 cells showed an increase in CIP2A expression after treatment with doxorubicin, while MCF-7 cells showed a decrease in CIP2A expression. The overexpression of CIP2A in MCF-7 cells overcame the inhibition of cell proliferation in response to doxorubicin treatment. CIP2A expression was not affected by wild-type or mutant p53. However, mutant p53 blocked doxorubicin-mediated CIP2A down-regulation in HCT116 cells. As a regulation mechanism of doxorubicin-mediated CIP2A expression, we showed that phosphorylated Akt was involved in the suppression of CIP2A expression.

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1. Introduction

The abnormal function of oncogenes and tumor-suppressor genes leads to cancer, and many such genes are being identified. One of these genes, the cancerous inhibitor of protein phosphatase 2A (CIP2A), is highly expressed in head and neck squamous cell carcinoma, colon cancer, gastric cancer and breast cancer [1–4]. CIP2A stabilizes the c-Myc protein by inhibiting its degradation mediated by protein phosphatase 2A (PPP2A) in cancer cells, and it is associated with malignant growth and aggressiveness [1,2]. The proto-oncogene c-Myc has been linked to a diverse range of cellular functions, such as cell cycle regulation, proliferation, and growth. Therefore, aberrant c-Myc signaling promotes cell transformation and tumor progression. Phosphorylation at serine 62 (S62) by extracellular-regulated kinase 1/2 (ERK1/2) stabilizes c-Myc [5,6]. Threonine 58 (T58) is phosphorylated by glycogen synthase kinase (GSK-3β) only after S62 is phosphorylated, and only T58 phosphorylation targets c-Myc for degradation [7]. PPP2A dephosphorylates c-Myc at S62, which leads to the destabilization of c-Myc [8]. CIP2A blocks this PPP2A-mediated c-Myc dephosphorylation pathway [9].

Doxorubicin, an anthracycline, is commonly used to treat many cancers, including breast, Hodgkin’s lymphoma, bladder, and stomach cancers; but a major problem is the occurrence of drug resistance. Doxorubicin induces increased expression of the apoptotic CD95 receptor, thus, inducing apoptosis, and CD95 receptor expression is regulated by p53 [10,11]. These results suggest that tumor-suppressor protein p53 would affect the cytotoxicity of doxorubicin [12,13]. It has also been reported that a lack of p53 leads to doxorubicin resistance due to a reduced induction of apoptotic cell death [14]. However, a detailed mechanism remains to be elucidated. In this study, we measured the effect of CIP2A on the doxorubicin-mediated inhibition of cell proliferation because CIP2A increases the proliferation of several cancer cells. Our findings uncover the mechanisms of CIP2A regulation by doxorubicin and CIP2A-mediated doxorubicin resistance.

2. Materials and methods

2.1. Reagents and plasmids

Plasmids encoding four p53 mutants (MDA-MB-231 (R280K), MDA-MB-468 (R273H), BT549 (R249S), and R175H) were kindly obtained from Dr. Bernard W. Futscher at the University of Arizona, USA [15]. The plasmid encoding CIP2A was a kind gift of Dr. Jukka Westermarck at the University of Turku and Abo Akademi University, Finland. Plasmids expressing dominant negative Akt (DN-Akt), dominant negative PI3K (DN-PI3K), constitutively active Akt...
(CA-Akt), and constitutively active PI3K (CA-PI3K) were obtained from Dr. Jeong-Hyung Lee at Kangwon National University, Korea. Doxorubicin was purchased from Calbiochem (Darmstadt, Germany). Cisplatin, cyclophosphamide, and paclitaxel were purchased from Sigma-Aldrich (St. Louis, MO, USA). SB203580, SP600125, U0126, and LY294002 were purchased from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, USA).

2.2. Cell culture

Human breast cancer cell lines MDA-MB-231 and MCF-7 were purchased from the American Type Culture Collection. MDA-MB-231 cells, MCF-7 cells, the human colon cancer cell line HCT116, and p53-deficient HCT116 cells [16] were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (WelGENE, Daegu, Korea) supplemented with 10% fetal bovine serum (HyClone, South Logan, USA) at 37 °C in a humidified 5% CO2 incubator.

2.3. Proliferation assay

For the proliferation assay, cells transfected with the CIP2A expression plasmid or CIP2A siRNA [4] were seeded in 12-well plates at a density of 3 × 104 cells per well and treated with the indicated concentration of doxorubicin. The number of viable cells was determined by counting cells 24 h after the doxorubicin treatment, and dead cells were excluded using trypan blue staining.

2.4. Immunoblot analysis

Total cell lysates were prepared 24 h after transfection with mutant or wild-type p53 or the CIP2A expression plasmid or CIP2A siRNA, mixed with 5× sodium dodecyl sulfate (SDS) sample buffer, and then sonicated for 15 s. The sonicated samples were heated at 95 °C for 5 min and separated electrophoretically on a 12% SDS–polyacrylamide gel. Subsequently, proteins were transferred onto a 0.45-μm nitrocellulose membrane (GE Healthcare, England, UK) for 2 h. The membrane was incubated with antibodies specific for CIP2A (Santa Cruz Biotechnology, CA, USA; cat. no. sc-80659), p53 (Santa Cruz Biotechnology, CA, USA; cat. no. sc-6243), phospho-Akt (Cell Signaling Technology Inc., MA, USA; cat. no. 9275S), and Akt (Santa Cruz Biotechnology, CA, USA; cat. no. sc-1619). The membrane was then incubated with anti-rabbit or anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (Assay Design, MI, USA) at room temperature for 2 h. The proteins were visualized using an enhanced chemiluminescent substrate (Thermo Fisher Scientific, UT, USA) and analyzed using a LAS3000 luminescent image analyzer (Fuji Film, Tokyo, Japan).

2.5. Reverse transcription-PCR

Total cellular RNA was extracted using the RNAiso plus kit (Takara, Shiga, Japan). A total of 3 μg of RNA was transcribed with 0.5 μg of random primer using reverse transcriptase (Fermentes, Ontario, Canada) at 42 °C, for 1 h. The followings primers were used for amplification: CIP2A, forward primer (5′-GGGAATTCCTT-GATCTCCTTCA-3′) and reverse primer (5′-CCCTCGAGCTAGAAG-CCTACTCCAT-3′), and β-actin, forward primer (5′-GTGGGGG-GCCCCCGACCA-3′) and reverse primer (5′-CTCCAATGTCA-GCGACGAT-3′). PCR was performed using 30 cycles of amplification. Each amplification cycle consisted of 30 s of denaturation at 94 °C, 30 s of annealing at 55 °C, and 30 s of extension at 72 °C, and PCR was performed using a PTC-100 from MJ Research Inc. (Waltham, MA, USA). The reaction mixtures of PCR were run at 1% agarose gel and photographed.

3. Results

3.1. Effect of CIP2A expression on the doxorubicin-mediated inhibition of breast cancer cell proliferation

Previously, it was shown that MDA-MB-231 cells deficient in CIP2A show a significant decrease in anchorage-independent growth, tumor volume, and proliferation [1]. In this study, we examined whether an increase in CIP2A expression was associated with sensitivity against the doxorubicin-induced inhibition of cell proliferation. MDA-MB-231 cells were first transfected with CIP2A, and the expression of CIP2A was confirmed using RT-PCR and immunoblot analysis (Fig. 1A). CIP2A was highly expressed at both the mRNA and protein levels. The overexpression of CIP2A resulted in a significant increase in the proliferation of breast cancer cells (Fig. 1B). To further confirm this result, MDA-MB-231 cells were transfected with CIP2A siRNA, and the expression of CIP2A was examined using immunoblot analysis (Fig. 1C). The level of CIP2A was markedly decreased by the CIP2A siRNA treatment and the knock-down of CIP2A resulted in a significant decrease in the proliferation (Fig. 1D). Then, we examined whether treatment with doxorubicin affected the expression level of CIP2A. MDA-MB-231 and MCF-7 cells were treated with doxorubicin, and the expression level of CIP2A was determined using RT-PCR and immunoblot analysis. CIP2A expression was transcriptionally and translationally increased in MDA-MB-231 cells but decreased in MCF-7 cells after doxorubicin treatment in a dose-dependent manner (Fig. 2A). To determine whether this difference in CIP2A expression was associated with sensitivity against the doxorubicin-induced inhibition of cell proliferation, the cell proliferation levels of both cells were
measured after doxorubicin treatment. MCF-7 cells were more susceptible to doxorubicin-mediated inhibition of cell proliferation than MDA-MB-231 cells (Fig. 2B). To determine whether the difference in sensitivity against doxorubicin was caused by the difference in the CIP2A expression level, MCF-7 cells were transfected with CIP2A and then treated with doxorubicin. Overexpression of CIP2A overcame the doxorubicin-mediated inhibition of cell proliferation (Fig. 2C). To further confirm this result, MDA-MB-231 cells were transfected with CIP2A siRNA to knock-down CIP2A expression and treated with 0.25, 0.5, and 1 μM concentration of doxorubicin for 24 h, and then viable cells were then counted. Data shown are representative of three independent experiments performed in triplicates. All values are represented as the means ± standard deviation. *P < 0.05 versus control. (E) MCF-7 cells were treated with the indicated concentration of drugs for 24 h and the level of CIP2A level was determined using immunoblot analysis. Cyclo, cyclophosphamide; Pac, paclitaxel.

3.2. Mutant p53 blocks doxorubicin-mediated CIP2A down-regulation in HCT116 cells

Because it has previously been shown that CIP2A is more highly expressed in breast cancer patients with a p53 mutation compared to patients with wild-type p53 [1] and that treatment with doxorubicin greatly decreased the expression level of CIP2A in MCF-7 cells containing wild-type p53 but increased the CIP2A expression level in MDA-MB-231 cells containing only mutant p53 (Fig. 2A), we determined the relationship between CIP2A and p53. First, to determine whether wild-type p53 was responsible for the decrease in CIP2A expression in response to doxorubicin treatment, p53-deficient HCT116 cells were transfected with wild-type p53 expression plasmid followed by treatment with doxorubicin. Doxorubicin decreased CIP2A expression in HCT116 cells similar to MCF-7 cells, whereas it increased CIP2A expression in p53-deficient HCT116 cells in a dose-dependent manner (Fig. 3A), similar to MDA-MB-231 cells. Thus, to determine whether wild-type p53 was responsible for the decrease in CIP2A expression in response to doxorubicin treatment, p53-deficient HCT116 cells were transfected with a wild-type p53 expression plasmid followed by treatment with doxorubicin. Doxorubicin increased CIP2A expression regardless of the presence of wild-type p53 (Fig. 3B). These results demonstrate that doxorubicin increases CIP2A expression through a wild-type p53-independent pathway.

Next, to determine whether mutant p53 was involved in the increase in CIP2A expression in response to doxorubicin treatment, p53-deficient HCT116 cells were transfected with mutant p53 because MDA-MB-231 cells only express mutant p53 (R280K),
The expression of mutant p53 did not affect the doxorubicin-mediated up-regulation of CIP2A expression (Fig. 4A). To determine the effect of other types of p53 mutants on CIP2A expression, p53-deficient HCT116 cells were transfected with various types of mutant p53, including R273H, R249S, and R175H. None of the mutant p53 proteins affected the doxorubicin-mediated up-regulation of CIP2A expression (Fig. 4B). Next, HCT116 cells were transfected with R273H, R249S, and R175H mutants. All mutant forms of p53 partially blocked doxorubicin-mediated CIP2A down-regulation (Fig. 4C). These findings imply that mutant p53 is able to act as a dominant negative in doxorubicin-mediated CIP2A down-regulation in HCT116 cells. Therefore, wild type p53 seems to be necessary but not sufficient for the CIP2A down-regulation in response to doxorubicin.

3.3. Akt is associated with regulation of CIP2A expression by doxorubicin

To elucidate which signal pathway was involved in the doxorubicin-mediated enhancement of CIP2A expression, p53-deficient HCT116 cells were pretreated with p38, JNK, MEK, or PI3K inhibitors and then treated with doxorubicin 24 h. The p38 MAPK and PI3K inhibitors blocked doxorubicin-induced CIP2A expression (Fig. 5A). To further confirm this, DN-PI3K, which mimics PI3K...
mediated inhibition of cell proliferation (Fig. 6C), indicating that in which CIP2A was overexpressed overcame the doxorubicin-level of Akt in breast cancer cells [19], we assessed the phosphor-
depression of Akt in response to doxorubicin treatment, whereas p53-deficient HCT116 cells showed an increase in the level of Akt phosphorylation in response to doxorubicin treatment. To this end, HCT116 cells were transfected with CA-Akt to acti-
vate doxorubicin-induced Akt suppression, CIP2A expression was assessed. CIP2A expres-
sion level than p53-deficient HCT116 cells (Fig. 6B). In addition, HCT116 cells showed Akt dephosphorylation in response to doxorubicin treatment of breast cancer cells [19], we assessed the phosphory-
lation level of Akt after doxorubicin treatment. HCT116 cells showed an increase in the level of Akt phosphorylation in response to doxorubicin treatment, whereas p53-deficient HCT116 cells showed a decrease in Akt phosphorylation (Fig. 5C). Therefore, we wondered whether Akt affected the level of CIP2A expression in response to doxorubicin treatment. To this end, HCT116 cells were transfected with DN-Akt to suppress doxorubicin-induced Akt activation, and CIP2A expression was assessed. CIP2A expression was increased in HCT116 cells transfected with DN-Akt. When p53-deficient HCT116 cells were transfected with CA-Akt to activate doxorubicin-induced Akt suppression, CIP2A expression was weakly suppressed (Fig. 5D). These results imply that Akt activation is associated with the doxorubicin-mediated regulation of CIP2A expression.

To assess whether doxorubicin-mediated increase of CIP2A expression in p53-deficient HCT116 cells was related with resistance to doxorubicin, firstly, levels of CIP2A expression in both cell lines were compared. HCT116 cells showed higher CIP2A expression level than p53-deficient HCT116 cells (Fig. 6A). HCT116 cells and p53-deficient HCT116 cells were then treated with varying concentrations of doxorubicin. Although HCT116 cells proliferated faster than p53-deficient HCT116 cells in the absence of doxorubi-
cin, the proliferation of p53-deficient HCT116 cells was not relatively decreased at the increasing concentration of doxorubicin compared with HCT116 cells (Fig. 6B). In addition, HCT116 cells in which CIP2A was overexpressed overcame the doxorubicin-mediated inhibition of cell proliferation (Fig. 6C), indicating that cells with an increase in CIP2A were more resistant to doxorubicin.

4. Discussion

Cancers acquire resistance to chemotherapeutic drug. Although a common feature of multidrug resistance is a net decrease in the intracellular accumulation of drugs as a consequence of enhanced drug efflux, other possible contributing mechanisms exist. For example, alterations in glutathione metabolism [20,21], differential oxygen free-radical susceptibility [22], increased DNA repair, and genetic alterations, including tumor-suppressor genes, onco-
genes, cell cycle regulator genes, and growth factor receptor genes, are associated with drug resistance. The oncogene CIP2A is highly expressed in human head and neck squamous cell carcinomas, colon cancer, gastric cancer [4,23], and breast cancer tissues, and it is involved in the clinical aggressiveness and malignant growth of tumors [1]. However, the role of CIP2A in chemotherapeutic drug resistance has not yet been established. We show here that the in-
crease in CIP2A expression is associated with doxorubicin sensitivity. Doxorubicin treatment decreased the expression of CIP2A in cells having wild-type p53, including MCF-7 and HCT116 cells, whereas it increased expression in cells having mutant p53, including MDA-MB-231 and p53-deficient HCT116 cells. Therefore, we examined whether p53 was associated with the doxorubicin-
mediated regulation of CIP2A expression. Our results showed that the doxorubicin-increased CIP2A expression was not regulated by wild-type p53 (Fig 3B). However, mutant p53 blocked the doxorubicin-mediated down-regulation of CIP2A expression in the presence of p53 (Fig 4C). It means that coexistence of mutant and wild-type p53 is involved in the regulation of CIP2A expression by the treatment of doxorubicin. Collectively, wild-type p53 is nec-
essary but not sufficient for the suppression of CIP2A expression by the treatment of doxorubicin.

In addition to a previous report showed that the depletion of CIP2A inhibited cell growth and clonogenic capability regardless of the presence of p53 [4], it is reported that CIP2A-positive and p53-immunopositive patients show a shorter cumulative 10-year overall survival rate than CIP2A-negative and p53-immunopositive groups in gastric cancer [3]. Our result also provides that doxorubicin-mediated increase of CIP2A expression in mutant p53-har-
boring tumor cells is associated with doxorubicin resistance, whereas tumor cells expressing wild-type p53 are susceptible in response to doxorubicin because of CIP2A down-regulation. In addition, breast cancer patients with mutant p53 show a higher expression level of CIP2A compared with patients with wild-type p53 [1]. However, in our study, mutant p53 did not increase CIP2A expression without doxorubicin treatment in vivo (Fig. 4A). There-
fore, further study on the regulation of CIP2A expression would provide an answer about the relationship between p53 and CIP2A expression and function in vivo.

Several reports have strongly suggested that Akt activation is related to doxorubicin resistance. Depending on the cell types, treatment of breast cancer cells with doxorubicin triggers a tran-
sient phosphorylation and activation or dephosphorylation and inactivation of Akt through activation of PI3K [24], and the expres-
sion of upstream Akt activators, including HER2 and HER3, poten-
tiates Akt phosphorylation, and the resulting Akt activation leads to drug resistance [25,26]. Because the expression of DN-Akt in HCT116 cells and the expression of CA-Akt in p53-deficient HCT116 cells blocked the effect of doxorubicin on the differential regulation of CIP2A expression (Fig. 5D), it is likely that Akt is in-
volved in the differential CIP2A expression in response to doxoru-
bicin treatment. In our system, HCT116 cells showed an increase in Akt phosphorylation after doxorubicin treatment. HCT116 cells, however, showed more sensitivity to doxorubicin-induced inhibi-
tion of cell proliferation than p53-deficient HCT116 cells, which showed Akt dephosphorylation in response to doxorubicin.
treatment (Fig. 5C). HCT116 cells showed down-regulation of CIP2A expression in response to doxorubicin treatment, and the overexpression of CIP2A in HCT116 cells overcame the doxorubicin-mediated inhibition of cell proliferation (Fig. 6B). These results imply that CIP2A expression is a more critical event in doxorubicin resistance than Akt phosphorylation.

It is known that doxorubicin-induced PI3K/Akt activation is linked to p53 activation and that the resulting activated p53 stimulates target gene expression [27]. Although the doxorubicin-PI3K-Akt-p53 pathway may regulate CIP2A expression, a p53-independent doxorubicin-PI3K-Akt-CIP2A signal pathway may exist because our data showed that the doxorubicin-regulated CIP2A up-regulation was not completely dependent on wild-type or mutant p53. It also remains to be elucidated how HCT116 cells and p53-deficient HCT116 cells show a differential Akt phosphorylation pattern and CIP2A expression in response to doxorubicin treatment. Our findings indicate that CIP2A inhibition is applicable to developing combination chemotherapy of doxorubicin and that p53 status is an important factor to be considered because mutant p53 inhibits down-regulation of CIP2A expression in response to doxorubicin.

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