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Opposing securin and p53 protein expression in the oxaliplatin-induced cytotoxicity of human colorectal cancer cells

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Abstract

Oxaliplatin, a clinical anticancer drug, has been used to treat colorectal cancer. Securin and p53 have been shown to regulate the cell cycle arrest and apoptosis. However, roles of securin and p53 on the oxaliplatin-induced cytotoxicity in human colorectal cancer cells remain unclear. Treatment with 1–10 μM oxaliplatin for 24 h induced the cell death, growth inhibition, and cell cycle arrest in RKO colorectal carcinoma cells. The phospho-p53 (Ser-15), total p53, and p21 proteins were elevated by oxaliplatin in RKO cells; conversely, oxaliplatin decreased the securin protein expression. The p53-functional RKO cells were higher on the cytotoxicity and cell cycle arrest at the G_1 and G_2/M phases than the p53-mutational SW480 cells after treatment with oxaliplatin. Oxaliplatin inhibited the securin protein expression in the p53-functional cells but not in the p53-mutational cells. The securin-wild type cells were more sensitive than the securin-null cells on the increases of cytotoxicity and sub-G_1 fractions following treatment with oxaliplatin. Nevertheless, oxaliplatin elevated the activation of p53 in both securin-wild type and securin-null cells. As a whole, it is the first time to demonstrate that oxaliplatin inhibits the securin protein expression via a p53-dependent pathway, and p53 and securin may modulate the oxaliplatin-induced cytotoxicity in human colorectal cancer cells.

Keywords: Colorectal cancer; Oxaliplatin; p53; Securin; Apoptosis; Cell cycle

1. Introduction

The disruption of balance between apoptosis and survival pathways participates in the pathogenesis of cancer and may be exploited for cancer therapy (Franke et al., 1997; Morgan and Kastan, 1997). p53, a key tumor suppressor, inhibits cell growth, promotes apoptosis in cancer cells, and subsequently prevents tumor development (Bates and Vousden, 1996; Hofseth et al., 2004; Levine, 1997). The activation of p53 is mediated by phosphorylation at various sites that confer different roles in the cellular stresses (Canman and Lim, 1998; Dumaz and Meek, 1999; Meek, 1999; Oda et al., 2000a,b). The physiologic events of p53 are mediated through its downstream genes (Bates and Vousden, 1996; Hofseth et al., 2004; Levine, 1997). For example, induction of the cyclin-dependent kinase inhibitor p21 by p53 is central to the cell cycle arrest (Agarwal et al., 1995; Bates and Vousden, 1996; Hofseth et al., 2004). Besides, the downstream proteins of p53-mediated apoptosis include BAX (Miyashita and Reed, 1995), NOXA (Oda et al., 2000a,b), and PUMA (Nakano and Vousden, 2001).

Securin is also known as pituitary-tumor transforming gene (PTTG), Cut2, Pds1, and Pimples in a variety of

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species (Dominguez et al., 1998; Funabiki et al., 1996; Nagao et al., 2004; Yamamoto et al., 1996; Zou et al., 1999). In normal condition, securin prevents abnormal sister chromatid segregation during mitosis and maintains genomic stability (Funabiki et al., 1996; Jallepalli et al., 2001; Stemmann et al., 2001; Yamamoto et al., 1996). Recently, it has been proposed that securin participated in the cellular DNA repair after irradiation (Nagao et al., 2004). Securin was expressed abundantly in most cancer cells (Dominguez et al., 1998; Pei and Melmed, 1997; Saez et al., 1999; Zou et al., 1999) that promoted the cell proliferation and tumorgenesis (Hamid et al., 2005; Zhang et al., 1999; Zou et al., 1999). However, securin induced aneuploidy (Christopoulou et al., 2003), genetic instability (Kim et al., 2005), and apoptosis (Hamid and Kakar, 2004). Therefore, the roles of securin in the regulation of cell death and survival are still inconclusive.

Oxaliplatin, an anticancer drug, is a third-generation platinum compound that has been used to treat colorectal cancer. The cisplatin-resistant cancers can still response to oxaliplatin (Ramanathan et al., 2003; Sandor, 1997; Tashiro et al., 1989; Wang and Lippard, 2005). Oxaliplatin induces the formation of intrastrand cross-links between two bases of DNA leading to the inhibition of replication and transcription of tumor cells (Chaney et al., 2005; Raymond et al., 2002). Oxaliplatin also induces apoptosis in cancer cells (Arango et al., 2004; Gourdier et al., 2002). It has been proposed that oxaliplatin is a good radiosensitizer to reduce the tumor size when combined with radiotherapy (Kjellstrom et al., 2005; Zhu and Willett, 2003). However, the roles of securin and p53 on the anticancer ability of oxaliplatin remain unclear.

The roles of securin and p53 proteins were investigated after treatment with oxaliplatin. Oxaliplatin increased the activation of p53 proteins in human colorectal carcinoma cells. The p53-functional cells were more susceptible to the cell death than the p53-mutational cells by treatment with oxaliplatin. Interestingly, the securin expression was markedly reduced by oxaliplatin. The loss of securin in colorectal carcinoma cells reduced the induction of cytotoxicity following oxaliplatin treatment. Our findings indicate that p53 and securin proteins modulate the cytotoxicity and cell cycle arrest in the oxaliplatin-treated cells.

2. Materials and methods

2.1. Chemicals and antibodies

Hoechst 33258, propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT), and the Cy3-labeled mouse anti-β-tubulin were purchased from Sigma Chemical (St. Louis, MO). Anti-p53 (DO-1), anti-extracellular signal-regulated kinase-2 (ERK-2) (C-14), and the FITC (fluorescein isothiocyanate)-labeled goat anti-mouse IgG antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-p53 (serine-15) (9284S) was purchased from Cell Signaling Technology, Inc. (Beverly, MA). Anti-p21 (Ab-1) was purchased from Oncogene Sciences Products (Boston, MA). Anti-securin (ab-3305) was purchased from Abcam (Cambridgeshire, UK). The Cy5-labeled goat anti-rabbit IgG was purchased from Amersham Biosciences (Little Chalfont Buckinghamshire, UK).

2.2. Cell lines and cell culture

RKO was a p53-wild type colorectal carcinoma cell line (Bhat et al., 1997). SW480 cells contained the double mutations of p53 gene, a G-to-A mutation in codon 273, and a C-to-T mutation in codon 309, that was established from the colorectal adenocarcinoma of a 50-year-old white male (Rodrigues et al., 1990; Weiss et al., 1993). The securin-wild type and -null HCT116 colorectal carcinoma cell lines were kindly provided by Dr. B. Vogelstein of Johns Hopkins University (Baltimore, MD). The cells were maintained in DMEM (Gibco, Life Technologies, Grand Island, NY) for RKO and SW480, and McCoy's 5A medium (Sigma Chemical) for HCT116. These complete media were supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and L-glutamine. The cells were cultured at 37 °C and 5% CO_2 in a humidified incubator (310/Thermo, Forma Scientific, Inc., Marietta, OH).

2.3. Cytotoxic MTT assay

The cell viability was determined by the MTT colorimetric assay as described (Kuo et al., 2004; Plumb et al., 1989). The cells were plated in 96-well plates at a density of 1×10^4 cells/well for 12–24 h (RKO for 12 h, SW480 for 18–20 h, HCT116 securin-wild type for 16–18 h, and HCT116 securin-null for 24 h) before treatment. At the end of treatment, the cells were washed with phosphate-buffered saline (PBS), and were re-cultured in complete medium for 2 days. Subsequently, the medium was replaced and the cells were incubated with 0.5 mg/ml of MTT in complete medium for 4 h. The intensity was measured at 565 nm using a plate reader for enzyme-linked immunosorbent assays. The relative percentage of cell viability was calculated by dividing the absorbance of treated cells by that of the control in each experiment.

2.4. Cell cycle analysis

For determination of cell cycle progression, the cells were analyzed by flow cytometry. The cells were plated at a density of 1×10^6 cells per 60-mm Petri dish in complete medium. At the end of treatment, the cells were collected and fixed with ice-cold 70% ethanol overnight at −20 °C. After centrifugation,

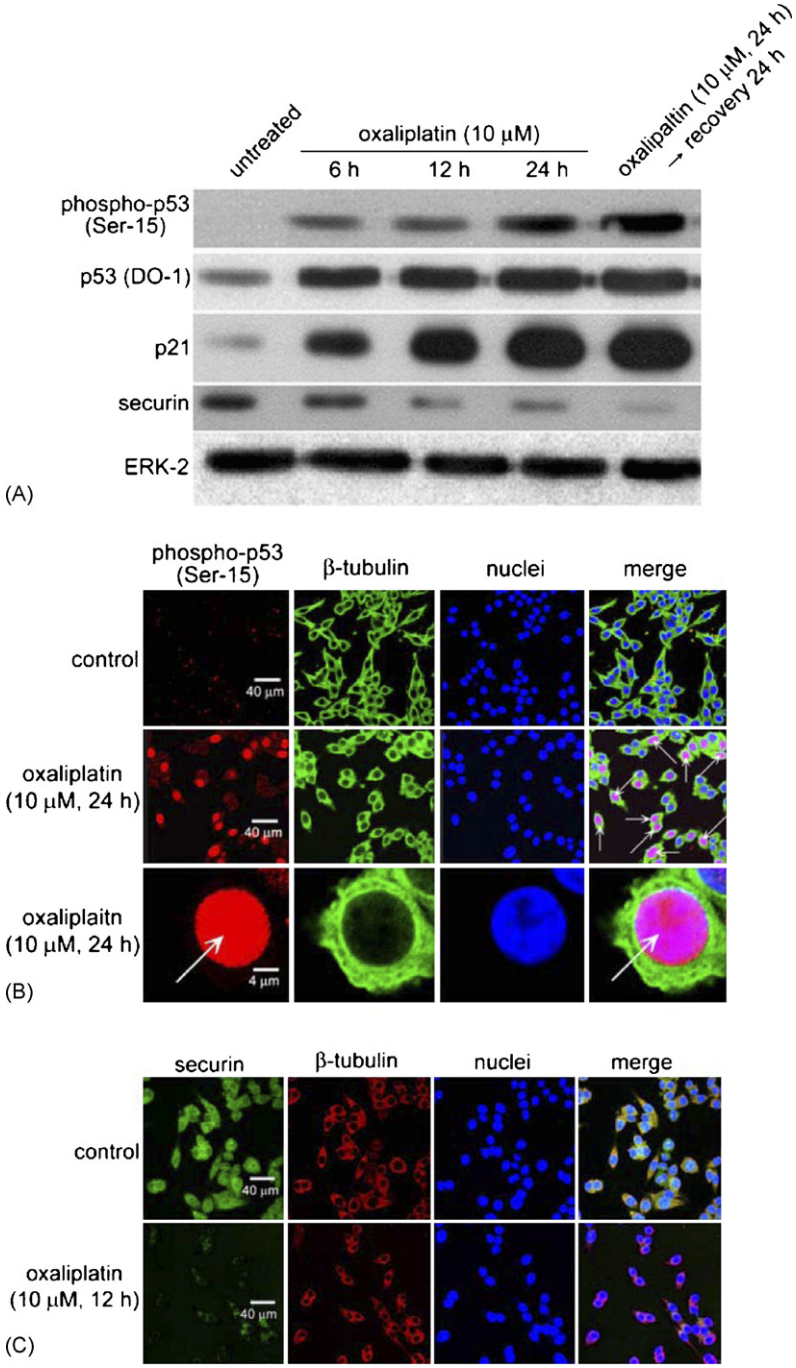

Fig. 1. Effects of oxaliplatin on the protein expression of p53, p21, and securin in colorectal carcinoma cells. (A) RKO cells were treated with 10 μM oxaliplatin for 6–24 h, or were treated with oxaliplatin (10 μM, 24 h) for 24 h recovery time. Representative immunoblot data were shown from one of three to five separate experiments with similar findings. (B) RKO cells were treated with or without 10 μM oxaliplatin for 24 h. The phospho-p53 (Ser-15) proteins displayed red fluorescence with goat anti-rabbit Cy5. Arrows indicated the location of phospho-p53 (Ser-15) proteins on the nucleus. (C) RKO cells were treated with or without 10 μM oxaliplatin for 12 h. The securin proteins displayed green fluorescence with goat anti-mouse FITC. The β-tubulin and nuclei were stained with the Cy3-labeled mouse anti-β-tubulin and Hoechst 33258, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.).

the cell pellets were treated with 4 μg/ml PI solution containing 1% Triton X-100 and 100 μg/ml RNase for 30 min. To avoid the aggregated cells, the cell solutions were filtrated by nylon membrane (BD Biosciences, San Jose, CA). The samples were analyzed in a BD system of flow cytometer (BD Biosciences) using CellQuest software. The percentage of cell cycle phases was quantified by using a ModFit LT software (BD Biosciences).

2.5. Immunofluorescence staining and confocal microscopy

To view the location and expression of p53 and securin proteins, the cells were subjected to immunofluorescence staining and confocal microscopy as described (Kuo et al., 2004). After treatment, the cells were washed with isotonic PBS (pH 7.4), and then fixed with 4% paraformaldehyde solution in PBS for 1 h at 37 °C. Then the cells were washed three times with PBS, and non-specific binding sites were blocked with PBS containing 10% FBS, 0.3% Triton X-100 for 1 h. Thereafter, the cells were incubated with rabbit anti-phospho-p53 (Ser-15) (1:250) or mouse anti-securin (1:20) antibodies in PBS containing 10% FBS for overnight at 4 °C. Then the cells were washed three times with 0.3% Triton X-100 in PBS and incubated with goat anti-rabbit Cy5 (1:250) or goat anti-mouse FITC (1:50) in PBS for 2–3 h at 37 °C. The β-tubulin was stained with the Cy3-labeled mouse anti-β-tubulin (1:50) for 30 min at 37 °C. Finally, the nuclei were stained with 2.5 μg/ml Hoechst 33258 for 30 min. The samples were immediately examined under a Leica confocal laser scanning microscope (Mannheim, Germany).

2.6. Immunoblot analysis

The total cellular protein extracts were collected as described (Chao et al., 2004; Kuo et al., 2004). Western analyses of phospho-p53 (Ser-15), p53 (DO-1), p21, securin, and ERK-2 were performed using specific antibodies. Equivalent amounts of proteins (20–60 μg/well) were subjected to electrophoresis using 10–12% sodium dodecyl sulfate-polyacrylamide gels. After electrophoretic transfer of proteins onto polyvinylidene difluoride membranes, the membranes were sequentially hybridized with primary antibody and followed with a horseradish peroxidase-conjugated second antibody (Santa Cruz Biotechnology, Inc.). The protein bands were visualized using the enhanced chemiluminescence detection system (Perkin-Elmer Life and Analytical Sciences, Boston, MA).

2.7. Statistical analysis

The data were analyzed using one- or two-way analysis of variance (ANOVA) test, and further post hoc tests using the statistic software of GraphPad Prism 4 (GraphPad software, Inc. San Diego, CA). A value of $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Oxaliplatin elevates the p53 protein expression but oppositely regulates the securin protein expression in colorectal carcinoma cells

Treatment with 10 μM oxaliplatin for 6–24 h time-dependently elevated the level of phospho-p53 (Ser-15), p53 (DO-1), and p21 proteins in RKO cells; however, the securin proteins were diminished (Fig. 1A). Moreover, the protein levels of phospho-p53 (Ser-15) p53 (DO-1), and p21 were persistently increased, and the securin protein expression was reduced by treating with oxaliplatin (10 μM, 24 h) for 24 h recovery in RKO cells (Fig. 1A). ERK-2 was used as an internal control in this study. The ERK-2 proteins were not altered following treatment with oxaliplatin (Fig. 1A). Consistently, the red fluorescence intensity (Cy5) exhibited by phospho-p53 (Ser-15) protein was increased after treatment with 10 μM oxaliplatin for 24 h (Fig. 1B). The phospho-p53 (Ser-15) proteins were highly concentrated on the nuclei in the oxaliplatin-exposed cells (Fig. 1B, arrows). The intensities of green fluorescence (FITC) exhibited by securin proteins were decreased after treatment with 10 μM oxaliplatin for 12 h (Fig. 1C).

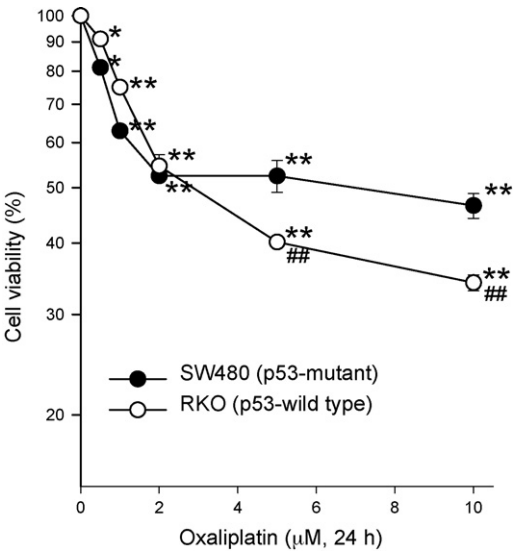

Fig. 2. Comparison of the cytotoxicity between the p53-functional and -mutational cells after treatment with oxaliplatin. RKO and SW480 cells were separately treated with 0–10 μM oxaliplatin for 24 h. The cell viability was estimated by MTT assay. Results were obtained from three experiments and the bar represents the mean ± S.E. $*p < 0.05$, $**p < 0.01$, comparison between the untreated and oxaliplatin-treated samples in RKO or SW480 cells using one-way ANOVA test. $\#\#p < 0.01$, comparison between RKO and SW480 cells from the untreated and oxaliplatin-treated samples using two-way ANOVA test.

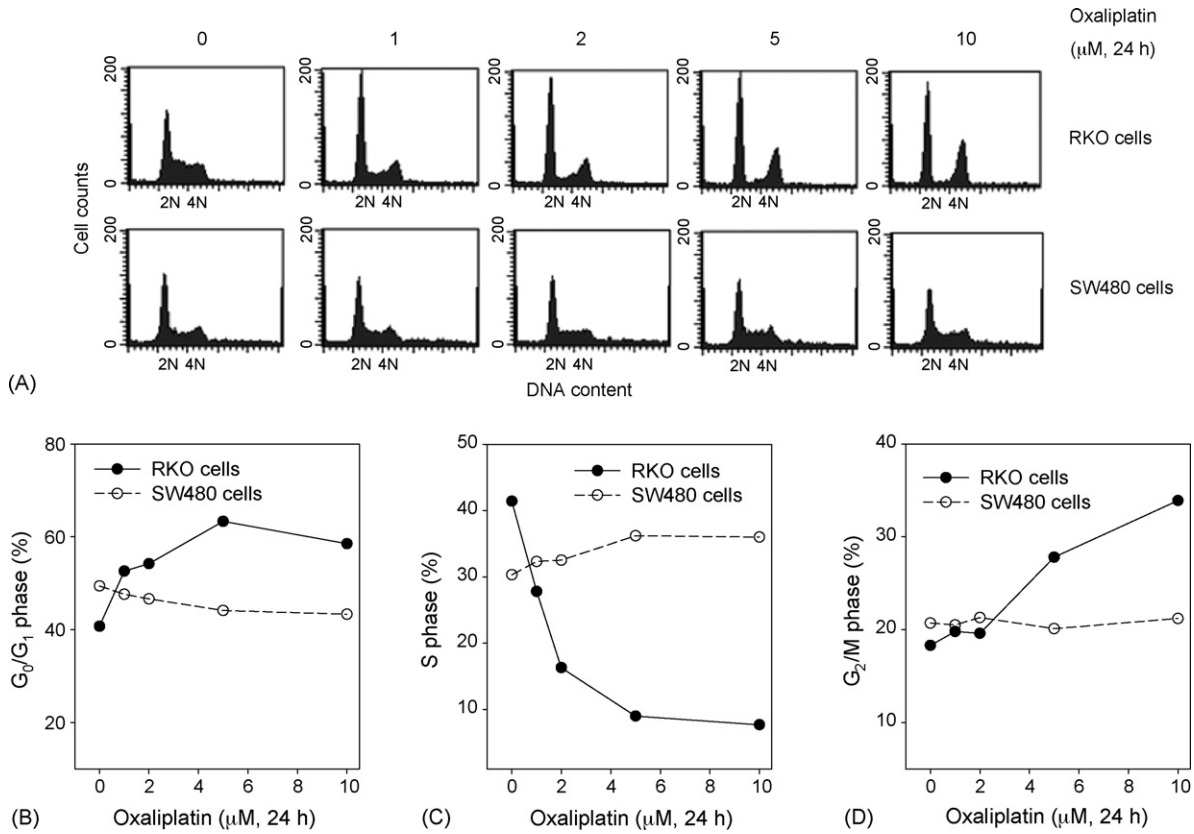

Fig. 3. Comparison of the cell cycle progression between the p53-functional and -mutational cells following treatment with oxaliplatin. (A) RKO and SW480 cells were separately treated with 0–10 μM oxaliplatin for 24 h. After treatment, the cells were trypsinized and then subjected to flow cytometry analyses. (B–D) The percentage of cell cycle phases was quantified by a ModFit LT software. The data represented the average values from three experiments.

3.2. Cytotoxicity and cell cycle arrest are higher in the p53 wild-type colorectal carcinoma cells after treatment with oxaliplatin

The cell viability after treatment with oxaliplatin was estimated by MTT assay. Oxaliplatin (1–10 μM for 24 h) concentration-dependently induced the cytotoxicity in both the p53-functional RKO and p53-mutational SW480 cells (Fig. 2). RKO cells were more susceptible than SW480 cells to the cytotoxicity after treatment with 5–10 μM oxaliplatin for 24 h (Fig. 2). Subsequently, RKO and SW480 cells were treated with oxaliplatin and the cell cycle progression was examined by flow cytometry. As shown in Fig. 3A, higher

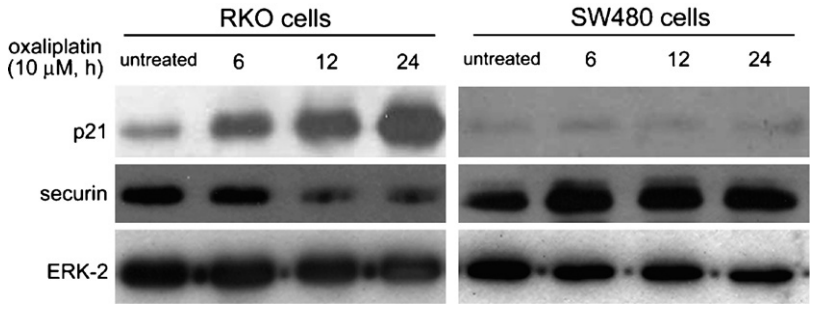

Fig. 4. Comparison of the protein expression of p21 and securin between the p53-functional (RKO) and -mutational cells (SW480) following treatment with oxaliplatin. The cells were treated with or without 10 μM oxaliplatin for 6–24 h. After treatment, the total protein extracts were prepared for Western blot analysis.

percentage of cell cycle arrest was seen in RKO cells than that of SW480 cells after treatment with 1–10 μM oxaliplatin for 24 h. The levels of G_1 and G_2/M phases were greater for RKO cells than SW480 cells following oxaliplatin treatment (Fig. 3B and D). In contrast, oxaliplatin markedly decreased the fraction of S phase in RKO cells; however, the level of S phase was not significantly altered by oxaliplatin in SW480 cells (Fig. 3C). To further investigate the effect of oxaliplatin on the cell proliferation in colorectal carcinoma cells, the cells were analyzed by cell growth assay. Oxaliplatin inhibited the cell growth ability in RKO cells (data not shown).

3.3. Oxaliplatin increases the p21 protein expression and inhibits the securin protein expression via a p53-dependent pathway

As shown in Fig. 4, p21 protein expression in the RKO cells was time-dependently increased after treatment with 10 μM oxaliplatin for 6–24 h; however, the p21 proteins were not altered by oxaliplatin in the SW480 cells. Moreover, the securin proteins were markedly reduced in the RKO cells but not in the SW480 cells following treatment with oxaliplatin (Fig. 4).

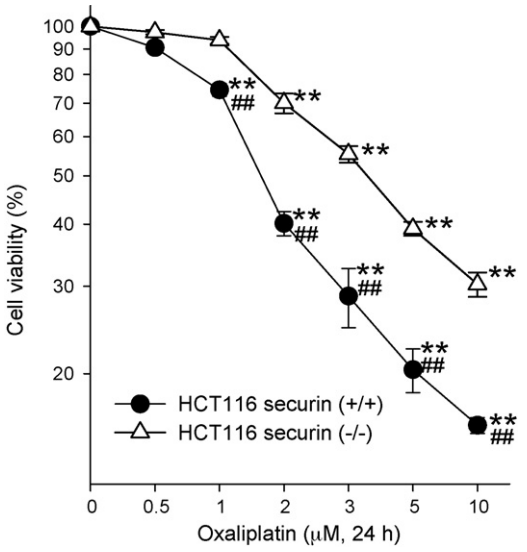

Fig. 5. Comparison of the cytotoxicity between the securin-wild type and -null HCT116 cells after oxaliplatin treatment. The cells were treated with 0–10 μM oxaliplatin for 24 h. The cell viability was estimated by MTT assay. Results were obtained from three experiments and the bar represented the mean ± S.E. *$p < 0.05$, **$p < 0.01$, comparison between the untreated and oxaliplatin-treated samples in the securin-wild type or -null cells using one-way ANOVA test. ##$p < 0.01$, comparison between the securin-wild type and -null cells from the untreated and oxaliplatin-treated samples using two-way ANOVA test.

3.4. Existence of securin in colorectal carcinoma cells increases the cytotoxicity and apoptosis following treatment with oxaliplatin

Treatment with 1–10 μM oxaliplatin for 24 h reduced the cell viability in both the securin-wild type and -null cells (Fig. 5). Moreover, the securin-wild type cells were more sensitive than securin-null cells on the increase of cytotoxicity after treatment with oxaliplatin (Fig. 5). The analysis of cell cycle indicated that oxaliplatin increased the G_2/M phases in both the securin-wild type and -null cells, but the securin-wild type cells were higher on the increase of sub-G_1 phase (apoptosis) than the securin-null cells by treated with 2–10 μM oxaliplatin for 24 h (Fig. 6). In addition, the phospho-p53 (Ser-15), p53 (DO-1), and p21 proteins were increased after exposure to 10 μM oxaliplatin for 3–24 h in both securin-wild type and -null cells (Fig. 7). The level of securin proteins was time-dependently diminished by oxaliplatin in the securin-wild type cells; however, the securin-null cells did not express securin proteins (Fig. 7).

4. Discussion

The blockade of survival pathways or the induction of apoptosis pathways by anticancer agents prevents the proliferation of tumor cells, which may be exploited for cancer therapy (Franke et al., 1997; Morgan and Kastan, 1997). p53 is a key protein that acts as a gatekeeper to control the cell cycle arrest and apoptosis (Agarwal et al., 1995; Bates and Vousden, 1996; Hofseth et al., 2004; Kuo et al., 2004; Levine, 1997). Therefore, the activation of p53 by anticancer agents may induce the cell cycle arrest and apoptosis in cancer cells leading to the inhibition of tumor progression. For example, doxorubicin and bleomycin activate the p53 protein expression to mediate apoptosis in cancer cells (Zhou et al., 2003). We have found that the levels of p53 proteins were increased in the oxaliplatin-treated colorectal carcinoma cells. Moreover, the loss of functional p53 reduced the cell death and cell cycle arrest in the oxaliplatin-exposed cells. p21, a downstream protein of p53, mediates the cell cycle arrest (Agarwal et al., 1995; Bates and Vousden, 1996; Hofseth et al., 2004). The level of p21 proteins was markedly elevated following treatment with oxaliplatin in colorectal carcinoma cells. These data suggest that the activation of p53 may mediate the cytotoxicity and cell cycle arrest after treatment with oxaliplatin in the human colorectal cancer cells.

In this study, it is the first time to prove that oxaliplatin inhibits the securin protein expression in human cancer cells. Treatment with anticancer agents including

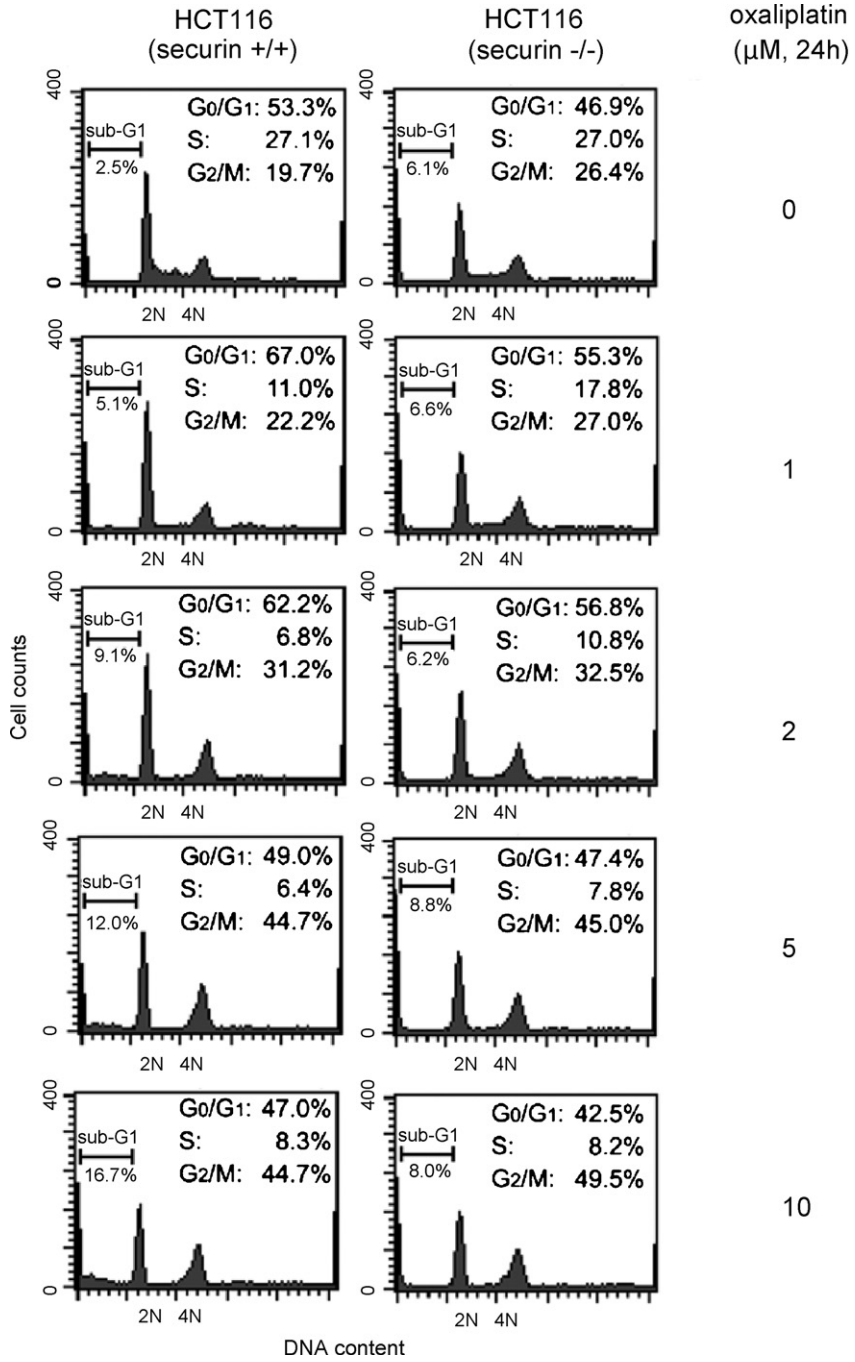

Fig. 6. Comparison of the cell cycle progression between the securin-wild and -null cells after treatment with oxaliplatin. The cells were treated with 0–10 μM oxaliplatin for 24 h. The cell cycle was analyzed by flow cytometry. The data represented the average values from three experiments.

ultraviolet, doxorubicin, and bleomycin also decrease the securin expression in cancer cells (Romero et al., 2004; Zhou et al., 2003). It has been shown that overexpression of securin induces apoptosis in human embryonic kidney cells (Hamid and Kakar, 2004). Furthermore, we found that the securin-wild type cells were more sensitive than the securin-null cells on the induction of cytotoxicity and apoptosis following treatment with oxaliplatin. These findings indicate that the existence of securin in colorectal cancer cells may increase the cytotoxicity and apoptosis after oxaliplatin treatment. Oxaliplatin, which is a DNA damage agent, induces the

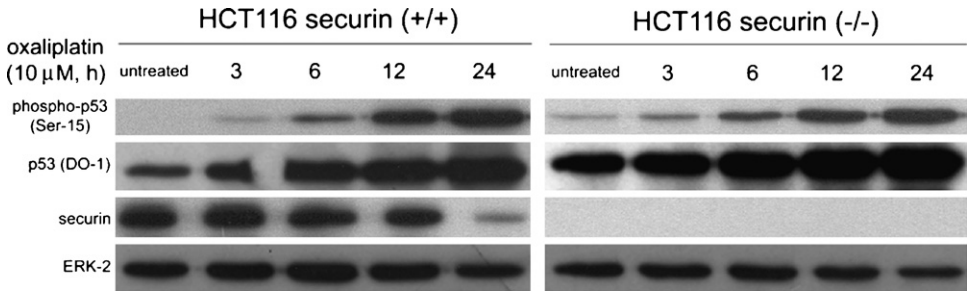

Fig. 7. Comparison of the activation of p53 and p21 between the securin-wild and -null cells after treatment with oxaliplatin. The cells were treated with or without 10 μM oxaliplatin for 3–24 h. Representative immunoblot data were shown from one of three separate experiments with similar findings.

formation of intrastrand cross-links between two adjacent guanine residues or a guanine and an adenine of DNA that results in the blockage of replication and transcription (Chaney et al., 2005; Raymond et al., 2002). However, oxaliplatin markedly diminished the securin protein expression in colorectal cancer cells. Recently, securin has been shown to participate in the cellular DNA repair (Nagao et al., 2004). Securin is important for DNA damage repair after exposure to ultraviolet, X-ray, and gamma-ray irradiation (Nagao et al., 2004). Moreover, securin prevents abnormal sister chromatid segregation during mitosis and maintains genomic stability, and its defects can result in chromosomal instability (Jallepalli et al., 2001; Yamamoto et al., 1996). Therefore, we propose that the loss of securin will decline cellular DNA repair ability and increases chromosome instability after treatment with oxaliplatin. Nevertheless, the precise mechanism and role of securin on the regulation of DNA repair in the oxaliplatin-exposed cells require further investigation.

Subsequently, p53 has been shown to inhibit the securin expression after treatment with anticancer drugs (Zhou et al., 2003). Securin proteins induce the p53 expression to mediate the BAX activation leading to apoptosis (Hamid and Kakar, 2004). In contrast, securin also interact with p53 to block the transcriptional activity of p53, which prevents the apoptosis (Bernal et al., 2002). We found that oxaliplatin diminished the securin protein expression in the p53-wild type cells but not the p53-mutational cells. The loss of functional p53 could not activate p21 protein expression following oxaliplatin treatment. Thus, we suggest that oxaliplatin reduces the securin proteins and induces the p21 proteins expression via a p53-depenedent pathway in colorectal carcinoma cells.

In conclusion, p53 and securin proteins can modulates the oxaliplatin-induced cytotoxicity in human colorectal cancer cells. Understanding the mechanisms by which p53 and securin pathways regulate the apoptosis and cell cycle arrest following treatment with oxaliplatin in cancer cells may contribute to the therapy of colorectal cancer.

Acknowledgements

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