Mitotic Arrest Deficient 2 Expression Induces Chemosensitization to a DNA-Damaging Agent, Cisplatin, in Nasopharyngeal Carcinoma Cells

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Abstract

Recently, mitotic arrest deficient 2 (MAD2)–mediated spindle checkpoint is shown to induce mitotic arrest in response to DNA damage, indicating overlapping roles of the spindle checkpoint and DNA damage checkpoint. In this study, we investigated if MAD2 played a part in cellular sensitivity to DNA-damaging agents, especially cisplatin, and whether it was regulated through mitotic checkpoint. Using nine nasopharyngeal carcinoma (NPC) cell lines, we found that decreased MAD2 expression was correlated with cellular resistance to cisplatin compared with the cell lines with high levels of MAD2. Exogenous MAD2 expression in NPC cells also conferred sensitivity to DNA-damaging agents especially cisplatin but not other anticancer drugs with different mechanisms of action. The increased cisplatin sensitivity in MAD2 transfectants was associated with mitotic arrest and activation of apoptosis pathway evidenced by the increased mitotic index and apoptosis rate as well as decreased Bcl-2 and Bax ratio and expression of cleaved poly(ADP-ribose) polymerase and caspase 3. Our results indicate that the MAD2-induced chemosensitization to cisplatin in NPC cells is mediated through the induction of mitotic arrest, which in turn activates the apoptosis pathway. Our evidence further confirms the previous hypothesis that spindle checkpoint plays an important part in DNA damage-induced cell cycle arrest and suggests a novel role of MAD2 in cellular sensitivity to cisplatin. (Cancer Res 2005; 65(4): 1450-8)

Introduction

Spindle checkpoint, or mitotic checkpoint, is a cellular monitoring system, which ensures that cell division does not take place until all the duplicated chromosomes align and attach to the spindle (1). Therefore, a functional mitotic checkpoint is essential for maintaining chromosomal stability. Recently, it has been suggested that chromosomal instability, which is found in a majority of human cancer cells, is a result of a defective mitotic checkpoint (2). In addition, increased chromosomal instability (or increased number of cells exhibiting gains or losses of chromosomes) is commonly observed in more advanced human cancers which usually show poor prognosis (3). These lines of evidence indicate that increased chromosomal instability, possibly impaired mitotic checkpoint control, may be associated with tumorigenesis as well as cancer progression.

Several regulators of the mitotic checkpoint have been identified and most of them are localized to the kinetochore, which is connected to both the chromosome and the spindle (1). One of them, mitotic arrest deficient 2 (MAD2), is thought to be a key component for a functional mitotic checkpoint because it is required for generating the "wait" signal in response to microtubule disruption (1). Deletion or down-regulation of *MAD2* leads to mitotic checkpoint inactivation and chromosomal instability (4–6). Down-regulation of MAD2 has also been reported in human cancers such as lung (7), breast (8), nasopharyngeal (9), and ovarian carcinomas (10). Recently, we have found that decreased MAD2 expression in nasopharyngeal carcinoma (NPC) cells is correlated with increased cellular sensitivity to certain microtubule disrupting anticancer drugs such as vincristine. Exogenous introduction of MAD2 leads to chemosensitization (11). These results indicate that MAD2 is not only a key factor in maintaining genomic stability but also an indicator of cellular sensitivity to certain types of anticancer drugs.

Recently, several studies have suggested a link between mitotic checkpoint and DNA damage response. For example, it has been found in both yeast and human cells that chemically induced DNA damage is able to activate the spindle checkpoint resulting in a mitotic arrest, which may be due to impairment on kinetochore attachment or function as a result of extensive DNA damage (12, 13). In addition, cytotoxicity of the DNA-damaging agent cisplatin is ascribed at least in part to the direct interruption of tubulin assembly (14). Recently, the MAD2-mediated DNA damage response has been correlated with sensitivity to certain anticancer drugs in p53-deficient colon cancer cells (15). The evidence that inactivation of MAD2 can override the DNA damage-induced mitotic block further indicates that this process may be regulated via a MAD2-dependent pathway (12, 13). Based on these results, we hypothesize that MAD2-mediated mitotic checkpoint may also play a part in cellular sensitivity to DNA-damaging agents. Therefore, in the present study, we used several NPC cell lines with differential MAD2 protein expression levels as well as NPC cells stably transfected with a MAD2 expression vector to investigate the role of MAD2 in cellular sensitivity to DNA-damaging agents, especially cisplatin, and the association of the MAD2-induced chemosensitization with mitotic checkpoint control.

Materials and Methods

Cell Lines and Cell Culture Conditions. Nine nasopharyngeal carcinoma cell lines [CNE3, SUNE1, CNE1 (16), CNE2 (17), HNE1, HONE1 (18), HK1 (19), TWO1, and TWO4 (20)] were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 2 mmol/L L-glutamine and

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5% (v/v) FCS at 37°C. The cultures were grown for a maximum of 10 passages. The immortalized nasopharyngeal cell line (NP69; ref. 21) was maintained in keratinocyte SFM medium (Life Technologies, Gaithersburg, MD) supplemented with penicillin G (100 units/mL), streptomycin (100 μg/mL), and gentamicin (50 μg/mL) at 37°C. Construction of the inducible MAD2 expression vectors and generation of stable CNE2-MAD2 transfectants were described in our previous studies (10, 11). The stable transfectants were maintained in both neomycin (800 μg/mL) and Zeocin (100 μg/mL). To induce MAD2 expression in the transfectants, 5 μmol/L of ponasterone A (Invitrogen) was added.

Colony-Forming Assay. Detailed experimental procedures have been described previously (11). Five hundred cells were plated in 6-well plates, resulting in 100 to 150 colonies per well after culturing for approximately 2 weeks. Cisplatin, taxol (Calbiochem, La Jolla, CA), vincristine, 5-fluorouracil (Both from David Bull Laboratories, Victoria, Australia), epirubicin (Pharmacia and Upjohn, Kalamazoo, MI), cyclophosphamid (ASTA Medica, Frankfurt, Germany), or doxorubicin (EBEWE, Unterach, Austria) were added 24 hours after plating, and colonies that consisted of more than 50 cells were scored and compared with the solvent-treated controls. Cells were also γ-irradiated using a γ-Cell 1000 Elite machine at dose rate of 14 Gy/min. The energy source was 137 Cs. Two wells were used for each drug concentration and five drug concentrations were tested for each experiment. Each experiment was repeated at least thrice and each survival curve showed the means and SDs of at least three independent experiments.

Western Blotting. Experiments were carried out as previously described (11). Briefly, cell lysates were prepared by suspending cell pellets in lysis buffer [50 mmol/L Tris–HCl (pH 8.0), 150 mmol/L NaCl, 1% NP40, 0.5% deoxycholate, and 0.1% SDS] including proteinase inhibitors (1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride). Protein concentration was measured using DC Protein Assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein (30 μg) were loaded onto a SDS-polyacrylamide gel for electrophoresis and then transferred on a polyvinylidene difluoride membrane (Amersham, Piscataway, NJ). The membrane was then incubated with primary antibodies for 1 hour at room temperature against MAD2, p55CDC, p53, p21 Waf1, Bcl-2, Bax (Santa Cruz Biotechnology, CA), caspase-3, and poly(ADP-ribose) polymerase (PARP, Cell Signaling Technology, Beverly, MA). After incubation with appropriate secondary antibodies, signals were visualized by enhanced chemiluminescence Western blotting system (Amersham). Expression of actin was also assessed as an internal loading control using a specific antibody (Santa Cruz Biotechnology). To study the effect of inhibitors on the cisplatin-induced apoptosis in CNE2MAD2 cells, caspase-9 inhibitor LEHD-FMK (10 or 20 μmol/L, Calbiochem) was added for an hour before cisplatin treatment. The cells were also treated with a Bcl-2 inhibitor HA14-1 (10 or 20 μmol/L, Calbiochem) together with cisplatin for different time points. Same volumes of solvent (DMSO) were added for the negative control experiments.

Cell Cycle Analysis. Flow cytometric analysis was done on an EPICS profile analyzer and analyzed using the ModFit LT2.0 software (Coulter) as described previously (22). Briefly, 5×10^5 cells were plated in culture medium and twenty-four hours later, different concentrations of cisplatin were added. The cells were harvested by trypsinization and then fixed in ice-cold 70% ethanol at indicated time points.

Determination of Apoptosis Rate. Cells were plated in Chamber slides and cisplatin was added 24 hours later. The cells were then fixed in ice-cold acetone and methanol (ratio, 1:1) at indicated time points, washed with PBS, and then stained with 4,6-diamidino-2-phenylindole for 5 minutes. The stained cells were examined under a fluorescence microscope and cells were considered to be apoptotic based on the appearance of nuclear fragmentation. A total of 500 cells were counted in five fields per sample. Percentage of apoptotic cells was calculated. Each experiment was repeated at least thrice and error bars indicate the SD.

Bromodeoxyuridine Staining. The bromodeoxyuridine incorporation rate was examined by avidin-biotin complex method using a Vectastain avidin-biotin complex kit (Vector Laboratories, Inc., Burlingame CA) and detailed experimental procedures have been described elsewhere (10). Each experiment was repeated thrice and error bars indicate the SD.

Determination of Mitotic Index. Detailed experimental procedures were described in our previous studies (9, 10). Briefly, cells were grown on Chamber slides and treated with cisplatin and collected at 24 hours post-exposure time. The cells were then fixed in cold methanol/acetone (1:1) for 5 minutes and stained with 4′,6-diamidino-2-phenylindole. The presence of condensed nuclear DNA indicates mitotic cells. To measure the mitotic index (percentage of viable cells arrested in mitosis), at least 500 cells were counted for each experiment using fluorescence microcopy and the data points represent the average results from three independent experiments.

3-(4,5-Dimethyl Thiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide Assay. The 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) proliferation assay kit was used according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN). Briefly, 3,000 cells were seeded in 96-well plates and then cultured in 5% FCS for 24 hours before any treatment was given. Before testing, 10 μL of MTT labeling reagent (5 mg/mL MTT in PBS) were added and the cells were incubated for a further 4 hours at 37°C. Then 100 μL of solubilizing reagent (10% SDS in 0.01 mol/L HCl) were added and the plate was incubated overnight at 37°C to dissolve the formazan crystals. The absorbance was measured at a wavelength of 570 nm on a Labsystem multiscan microplate reader (Merck Eurolab, Dietikon, Schweiz). Each time point was done in triplicate wells and each experiment was repeated at least twice. Each data point represented the mean and SD.

Results

Increased MAD2 Expression Confers Cellular Sensitivity to Cisplatin. Previously, we found that high percentage of NPC cell lines showed decreased MAD2 expression, which was associated with a defective mitotic checkpoint control (9). Ectopic expression of MAD2 in one of the NPC cell lines, CNE2, led to increased chemosensitivity to a microtubule disrupting agent, vincristine (11). In the present study, we investigated if MAD2 expression was associated with cellular sensitivity to DNA-damaging anticancer agents in nine NPC cell lines with differential MAD2 expression levels. The expression of MAD2 was also examined in a HPVE6/E7 immortalized nasopharyngeal epithelial cell line NP69 (21) as a control for nonmalignant cells. As shown in Fig. 1*A*, six NPC cell lines (66.7%) showed relatively low MAD2 levels compared with the nonmalignant cell line NP69, which confirms our previous hypothesis that down-regulation of MAD2 is a comment event in NPC cells (9). MAD2 protein level was the lowest in CNE2, CNE3 and TW01 cells but it was the highest in the CNE1, HONE1, and 915 cell lines. Colony forming assays on the cellular sensitivity to DNA-damaging agents cisplatin, carboplatin, and γ-irradiation showed that the three cell lines with low MAD2 protein expression (*dotted lines*) were more resistant than the ones with relatively high MAD2 expression (*solid lines*), especially to cisplatin (Fig. 1*B-D*).

To further investigate if MAD2 played a direct role in cellular sensitivity to DNA-damaging agents, we did colony forming assay on two stable MAD2 transfectants generated from CNE2 cells in a previous study (CNE2MAD2-C1 and CNE2MAD2-C2; ref. 11). Previously, the exogenous MAD2 expression was introduced in CNE2 cells by cotransfecting a MAD2 expression plasmid driven by an ecdysone-inducible promoter [pIND(SP1)-MAD2] and the plasmid expressing the heterodimeric ecdysone receptor (pVgRXR; ref. 10). Because ectopic MAD2 expression was inducible in the transfectants by ponasterone A (5 μmol/L), we first examined the MAD2 expression in the presence and absence of ponasterone A. As shown in Fig. 2*A*, increased MAD2 protein was found in the CNE2MAD2 transfectants (C1 and C2) after exposure to

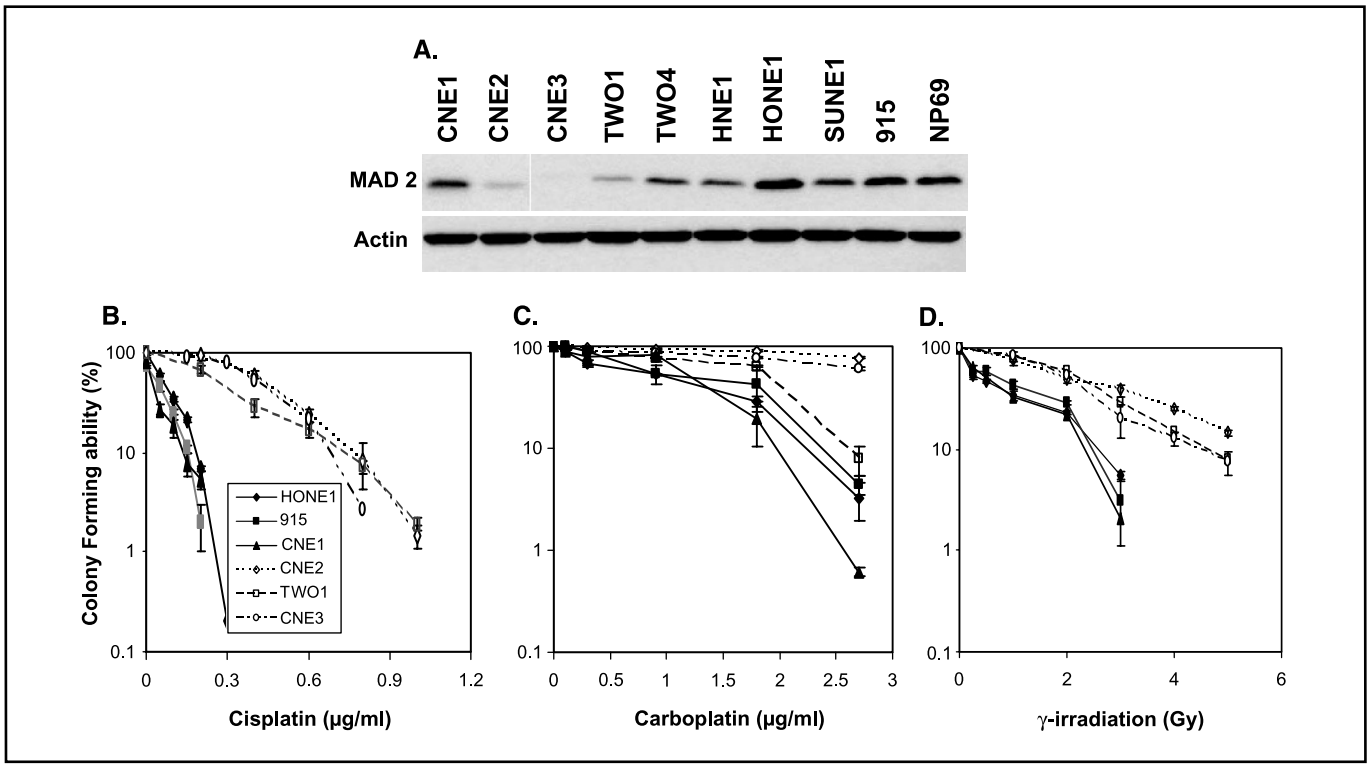


Figure 1. Increased MAD2 expression confers sensitivity to cisplatin. *A,* MAD2 expression in nine NPC cell lines CNE1, CNE2, CNE3, TWO1, TWO2, HNE1, HONE1, SUNE1, and 915 analyzed by Western blotting. The immortalized nasopharyngeal epithelial cell line NP69 was used as a nonmalignant cell control. Colony-forming ability of NPC cell lines with high and low levels of MAD2 protein levels after exposure to cisplatin (*B*), carboplatin (*C*), and γ-irradiation (*D*). Note that cells with high levels of MAD2 protein are more sensitive to these three agents.

ponasterone A for 24 hours (indicated as +P) compared with the untreated cells (indicated as −P), suggesting a successful introduction of MAD2 in CNE2 cells. We also found that there was no significant difference in cell proliferation rate between the cells treated with ponasterone A and the untreated cells showed by similar bromodeoxyuridine incorporation rates (Fig. 2*B*, $P > 0.05$), indicating that ponasterone A alone did not affect growth rate in the transfectants.

We then studied the cellular sensitivity of the ponasterone A treated and untreated cells to a range of DNA-damaging anticancer agents including cisplatin, carboplatin, cyclophosphamide, melphalan as well as γ-irradiation using colony-forming assay. As shown in Fig. 2*C*, after exposure to these agents, colony forming ability of the CNE2MAD2 cells was lower in the presence (*dotted lines*) than in the absence (*solid lines*) of ponasterone A. The most significant difference in cellular sensitivity between the cells with ectopically expressed MAD2 and the controls was observed after exposure to cisplatin. For example, the MAD2 overexpressing cells (CNE2-C1 and CNE2-C2) were 13.9- and 4.3-fold more sensitive to cisplatin, respectively, than the cells with low levels of MAD2 expression compared with their IC_{50} concentrations ($P < 0.001$; $IC50_{CNE2\text{-}MAD2\text{-}C1,\ -P}$ = 0.459 ± 0.05 μg/mL; $IC50_{CNE2\text{-}MAD2\text{-}C1,\ +P}$ = 0.033 ± 0.003 μg/mL; $IC50_{CNE2\text{-}MAD2\text{-}C2,\ -P}$ = 0.26 ± 0.04 μg/mL; $IC50_{CNE2\text{-}MAD2\text{-}C2,\ +P}$ = 0.06 ± 0.004 μg/mL). In contrast, when additional anticancer drugs with different mechanisms were tested, as shown in Fig. 2*D*, no significant difference in cellular sensitivity was observed between the cells with high (*dotted lines*) and low (*solid lines*) levels of MAD2 protein. These drugs include anthracyclines (doxorubicin and epirubicin) and antimetabolite (5-fluorouracil). These results indicate that ectopic MAD2 expression in CNE2 cells leads to increased sensitivity to DNA-damaging agents, especially cisplatin, in CNE2 cells.

MAD2-Induced Sensitization to Cisplatin Is Associated with a Mitotic Arrest. MAD2 is a key factor in regulating mitotic arrest. Overexpression of MAD2 in cells sufficiently induces metaphase arrest, whereas microinjection of anti-MAD2 antibody or depletion of MAD2 blocks this arrest (4–6). In light of this, we next studied if the MAD2-induced sensitization to DNA-damaging agents was associated with induction of cell cycle arrest. Because the MAD2-induced sensitivity to cisplatin was most evident, we then focused this study on cisplatin. First, we examined mitotic index after exposing to cisplatin in the MAD2 overexpressing cells and the parental CNE2 cells. As shown in Fig. 3*A*, the number of cells arrested in mitosis (representative morphology shown in *1* and also *dotted arrows* in *3*) as well as the number of cells undergoing apoptosis (representative photo shown in *1* and also *solid arrows* in *3*) in response to cisplatin treatment were studied. As shown in Fig. 3*B*, after exposure to cisplatin and ponasterone A for 24 hours (*solid columns*), the percentage of mitotic arrested cells was increased in the CNE2MAD2 transfectants in a cisplatin dose-dependent manner (0.5 and 1.0 μg/mL) compared with the untreated controls (*open columns*). Further cell cycle analysis also showed (Fig. 3*C*) that there was an S-phase block in the cells treated with cisplatin regardless of presence or absence of ponasterone A at 24 hours time point, especially after exposure to higher dose of cisplatin (1.0 μg/mL, *dotted arrows*). We did not observe any notable difference in the percentage of G_2-M phase

cells between the cells cultured in the presence and absence of ponasterone A at either 24 or 48 hours time points (representative results were shown as "Untreated control" at 24 hours time point in Fig. 3*C*). The cisplatin-induced S-phase arrest in CNE2 cells with low levels of MAD2 was consistent with previous studies (23) as well as the results generated in this study on CNE1 and 915 cells at earlier time point of 24 hours (Fig. 3*D*, *dotted arrows*). These results indicate that the cisplatin-induced S-phase block in NPC cells is independent of MAD2 levels. However, a G_2-M phase block was only observed in the CNE2MAD2 cells exposed to both ponasterone A and high dose of cisplatin (1.0 μg/mL) at later time point (48 hours, Fig. 3*C*, *solid arrows*). In addition, CNE1 and 915 cells which also had relatively high levels of MAD2 protein expression, showed a G_2-M arrest after exposure to cisplatin at later time point of 48 hours (Fig. 3*D*, *solid arrows*). These results indicate that the cisplatin-induced mitotic arrest is associated with high levels of MAD2 expression in NPC cells, especially at higher dose.

MAD2 Expression Promotes Cisplatin-Induced Apoptosis. It has been suggested that the cytotoxicity of many commonly used anticancer drugs is a result of prolonged cell cycle arrest, which leads to activation of apoptosis pathway (24).Therefore, we next investigated if the cisplatin-induced mitotic arrest in MAD2 overexpressing cells was associated with cell growth arrest and apoptosis in the MAD2 overexpressing cells by exposure to three doses of cisplatin (0.25, 0.5, and 1.0 μg/mL). Using bromodeoxyuridine incorporation assay, we first showed a decreased cell proliferation rate in the cells treated with both ponasterone A and cisplatin (Fig. 4*A*, *filled columns*) compared with the untreated cells (*open columns*). The growth arrest occurred in a cisplatin dose-dependent manner as more significant difference was observed with increased cisplatin doses ($P < 0.001$ after exposure to 1.0 μg/mL cisplatin), indicating that cisplatin treatment was sufficient to induce cell growth arrest in the MAD2 overexpressing cells. We then examined apoptosis rate between cells expressing high or low levels of MAD2 after exposure to cisplatin. As shown in Fig. 4*B*, after exposure to three doses of cisplatin, the apoptosis rate in the parental CNE2 cells was not significantly different between the cells treated with both ponasterone A and cisplatin compared with the cells treated with cisplatin alone ($P > 0.05$). In contrast, increased number of apoptotic cells was observed in the MAD2 transfectants expressing exogenous MAD2 protein (+P) in a dose- and time-dependent manner compared with the cells with low MAD2 protein levels (−P). For example, the number of cells showing nuclear fragmentation in the MAD2-C1 and MAD2-C2 cells

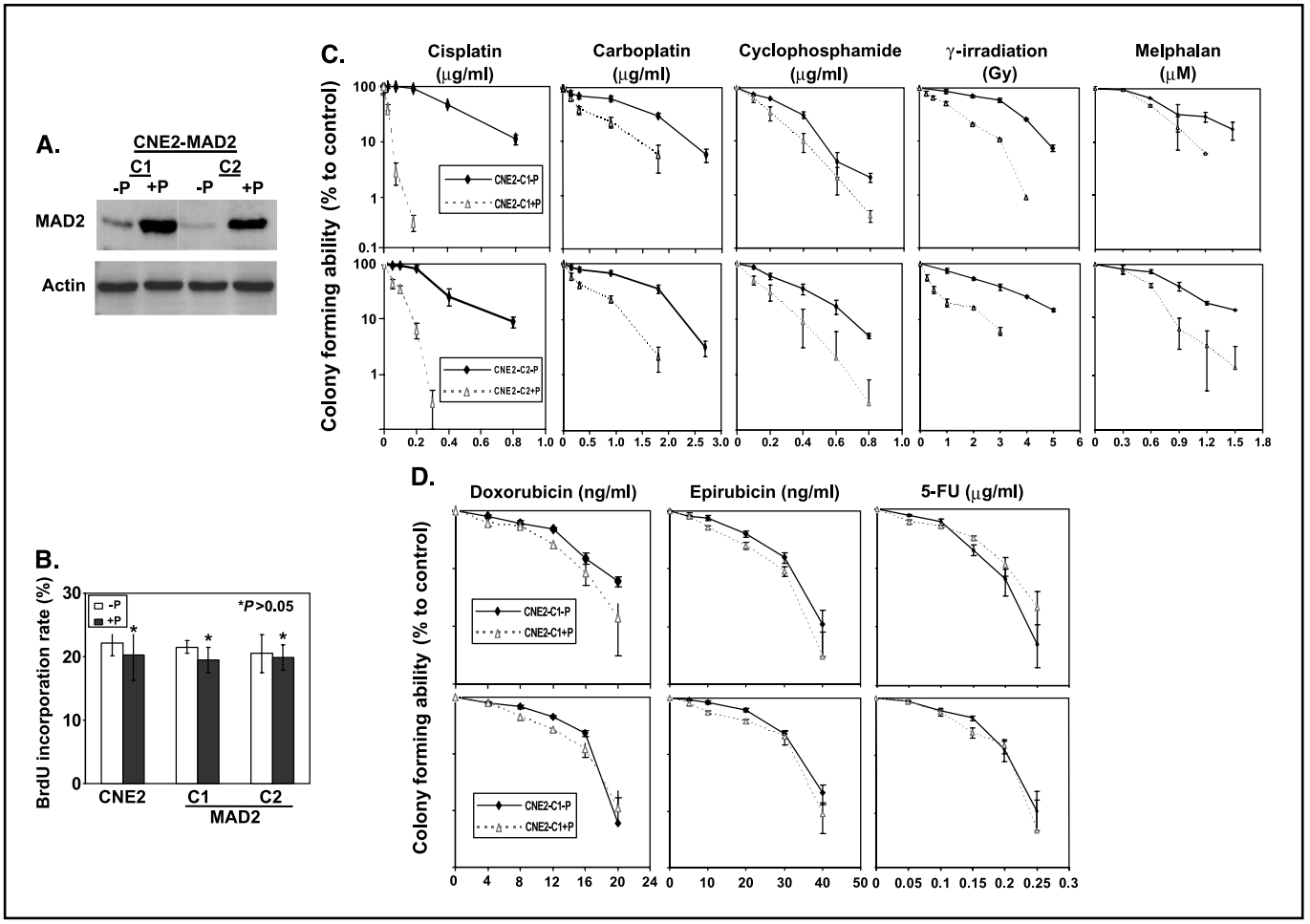


Figure 2. Ectopic MAD2 expression leads to increased sensitivity to DNA-damaging agents. *A,* MAD2 expression in stable CNE2 transfectants cultured in the presence (+P) and absence (−P) of ponasterone A for 24 hours. *B*, bromodeoxyuridine (*BrdU*) incorporation rate between the cells cultured in the presence and absence of ponasterone A. *C,* colony-forming ability of CNE2 cells expressing exogenous MAD2 protein (*dotted lines*) and the controls (*solid lines*) after continuous exposure to five DNA-damaging anticancer drugs. *D,* colony-forming ability of CNE2 cells expressing exogenous MAD2 protein (*dotted lines*) and the controls (*solid lines*) after continuous exposure to doxorubicin, epirubicin, and 5-fluorouracil. Note that higher MAD2 expression is associated with increased sensitivity to DNA-damaging agents but not to other anticancer drugs with different mechanisms of action. *Columns* and *points,* three independent experiments; *bars,* SD.

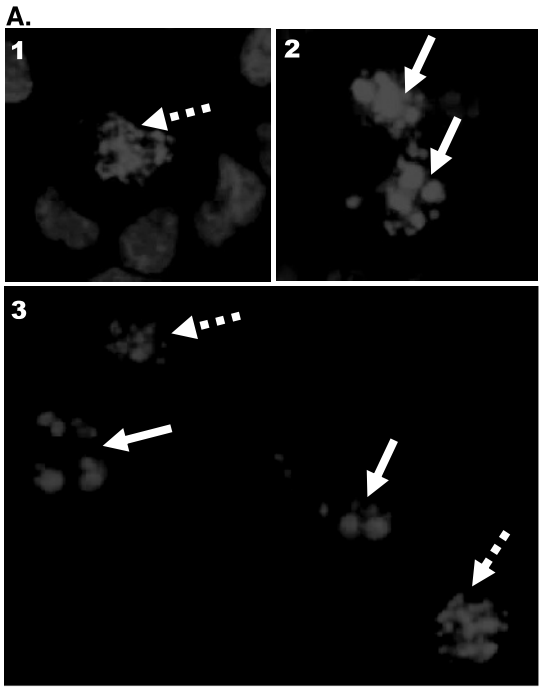
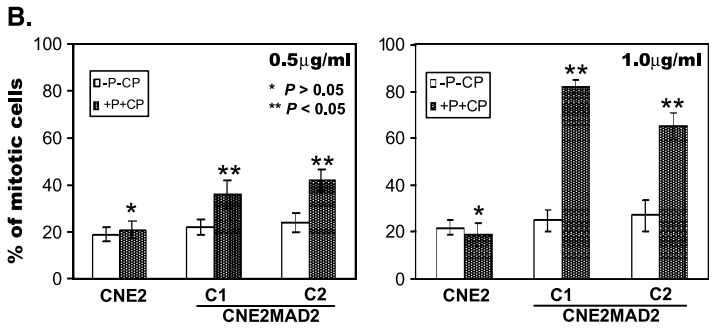
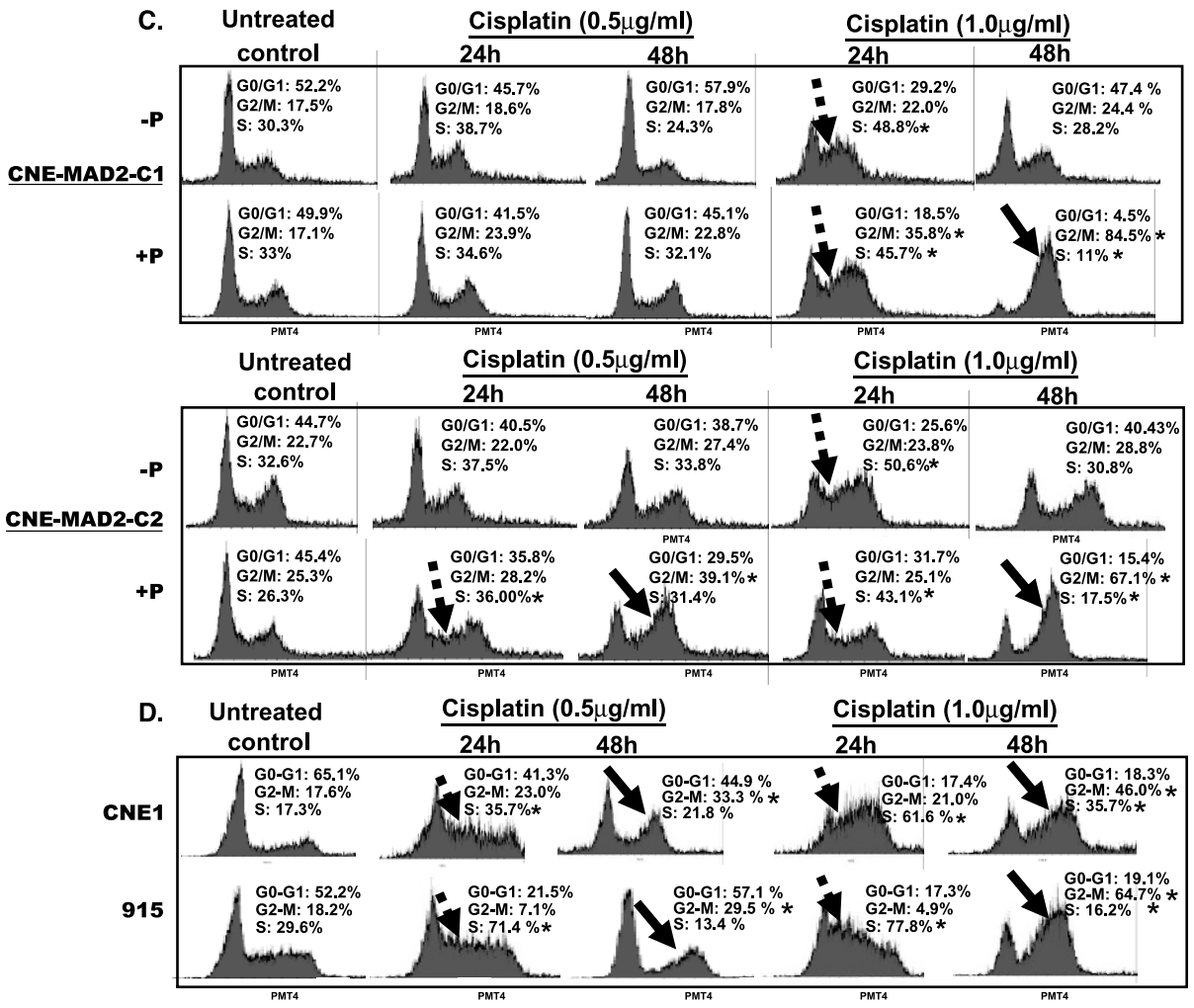


Figure 3. Exogenous MAD2 expression in CNE2 cells leads to cisplatin-induced mitotic arrest. *A,* representative photos of morphologic difference between mitotic (*1*) and apoptotic (*2*) cells. Photos were taken under ×400 magnifications. Note that cells showing nuclear fragmentation (*solid arrows*) are considered undergoing apoptosis, whereas cells showing chromosome condensation (*dotted arrows*) are considered as mitotic arrested cells. *B,* mitotic index of CNE2 transfectants expressing high (*solid columns*) or low (*open columns*) levels of MAD2 protein after exposure to cisplatin (0.5 and 1.0 μg/mL). Parental CNE2 cells were tested as a control and two-tailed Student's *t* test was used to analyze the difference in percentage of mitotic cells between the cells with high (+P) and low (−P) levels of MAD2 under same treatment conditions. *, $P < 0.05$ was considered as statistically significant. *C,* cell cycle distribution after exposure to cisplatin in CNE2 cells expressing high (+P) or low (−P) levels of MAD2 protein. *Solid arrows,* G_2-M cell cycle arrest; *dotted arrows,* S-phase arrest. *D,* cisplatin-induced cell cycle alteration in CNE1 and 915 cells. Note that cisplatin treatment leads to accumulation of S-phase cells in all cell lines at 24 hours post-exposure time and G_2-M arrest at 48 hours time point in the cells with increased MAD2. *Columns,* three independent experiments; *bars,* SD.





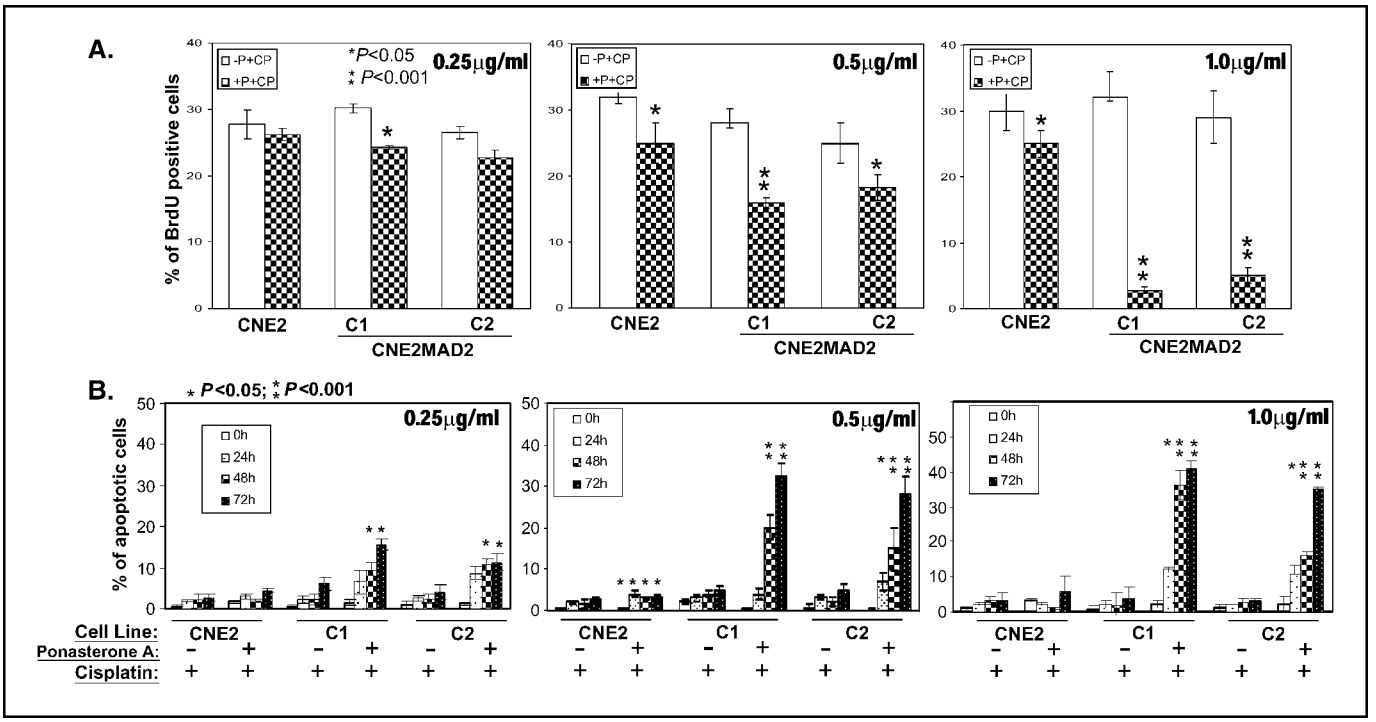


Figure 4. Exogenous MAD2 expression induces cell growth arrest and apoptosis in response to cisplatin treatment. Bromodeoxyuridine (*BrdU*) incorporation rate (*A*) and % cells undergoing apoptosis (*B*) in CNE2 transfectants cultured in the presence (+P) or absence (−P) of ponasterone A after exposure to cisplatin for 24 hours. Parental cells were tested as a control. Measurement of *BrdU* incorporation rate was described in Materials and Methods, and criteria for determination of apoptotic cells was described in Fig. 3*A* legend. Two-tailed Student's *t* test was used to analyze the difference in percentage of *BrdU*-positive cells and apoptotic cells between the cells with high (+P) and low levels (−P) of MAD2 under same treatment conditions. *, $P < 0.05$ was considered as statistically significant. Note that after exposure to cisplatin, cells with higher MAD2 expression show decreased cell proliferation and increased apoptosis rate. *Columns,* three independent experiments; *bars,* SD.

was significantly higher in the cells cultured in both cisplatin and ponasterone A than the cells treated with cisplatin alone, especially at 72 hours post-exposure time ($P < 0.001$). These results in combination with the data generated from previous sections indicate that the cisplatin-induced apoptosis in MAD2 overexpressing cells is associated with mitotic arrest and decreased cell proliferation rate.

MAD2-Induced Chemosensitization Is Associated with Activation of the Apoptosis Pathway. To further verify that the MAD2-induced sensitization to cisplatin was a result of increased apoptosis, we next studied the expression levels of several key regulators of the apoptosis pathway. CNE2MAD2 cells were treated with both cisplatin and ponasterone A for 16, 24, 36, and 48 hours and MAD2 protein levels were measured. The untreated parental line CNE2 was used as a control. As shown in Fig. 5, MAD2 protein levels were much higher in the ponasterone A–treated CNE2MAD2-C1 cells compared with the controls, indicating again a successful ectopic expression of MAD2 in the transfectants. p55CDC has been suggested to be a cofactor of MAD2 in regulating mitotic checkpoint and up-regulation of p55CDC has been shown to be associated with apoptosis (25). In the present study, we observed a slight increase in p55CDC levels at early time points (16 and 24 hours) in the MAD2-expressing cells, indicating a possible involvement of p55CDC in the induction of apoptosis in CNE2MAD2 cells. Because p53 is a key cell cycle regulator in response to DNA damage and induction of apoptosis, we next examined p53 protein level in the treated and untreated cells. We found that p53 protein level increased in both ponasterone A treated and untreated cells at later time points (i.e., 36 hours). It has been reported that a p53 mutation is present in this cell line (26). It is possible that the delayed induction of p53 protein in both cell types may not be due to its specific response to cisplatin-induced DNA damage. In contrast, increased phosphorylation of Bcl-2, which inactivates this protein (27), and up-regulation of Bax were observed in the MAD2 overexpressing cells, especially at later time points (36 and 48 hours). In addition, the cleaved forms of PARP and caspase 3, which are indicators of cells undergoing apoptosis (28, 29), were observed in the MAD2 overexpressing cells only at later time points when significant number of cells showed morphologic changes associated with apoptosis (Fig. 4*B*). Similar results were observed in CNE2MAD2-C2 cells (data not shown). These results further support the hypothesis that MAD2-induced sensitization to cisplatin is mediated through activation of the apoptosis pathway in CNE2 cells.

Effect of Bcl-2 and Caspase Inhibitors on MAD2-Induced Apoptosis. To further confirm the role of Bcl-2 and Bax in the MAD2-induced apoptosis, we then treated the CNE2MAD2 cells with HA14-1, a small molecule Bcl-2 inhibitor (30). This cell-permeable nonpeptidic ligand of the hydrophobic Bcl-2 surface pocket interferes with the ability of Bcl-2 to block the actions of proapoptotic Bcl-2 family members (e.g., Bax and Bak) and, in doing so, promotes apoptosis either by itself or in combination with cytotoxic agents (31). To study if HA14-1 could enhance the MAD2-induced apoptosis in CNE2 cells, we treated the CNE2MAD2-C1 cells with ponasterone A, HA14-1 and cisplatin for different period of time and measured the expression of cleaved caspase 3 and PARP as well as cell viability. As shown in Fig. 6*A*, the expression of cleaved PARP was higher in the cells treated with HA14-1 which was also associated with increased expression levels of the cleaved

caspase 3 compared with the cells treated with cisplatin and ponasterone A only. The increased cleaved PARP and caspase 3 was associated with decreased cell viability in the MAD2 expressing cells in a HA14-1 dose-dependent manner ($P < 0.05$ compared with the cells without HA14-1 treatment; Fig. 6*B*), indicating that inhibition of Bcl-2 in the cells with high levels of MAD2 led to increased sensitivity to cisplatin-induced cell death. To further address the significance of the apoptosis pathway in the MAD2-induced sensitization, we then treated the cells with a specific caspase 9 inhibitor, LEHD-FMK, and examined its effect on the cisplatin-induced apoptosis. As shown in Fig. 6*C*, in contrast to the effect of HA14-1, we found that the LEHD-FMK treatment resulted in a decreased expression of cleaved PARP and caspase 3 proteins compared with the cells treated with cisplatin and ponasterone A only, indicating the suppressive effect of LEHD-FMK on the apoptosis pathway. MTT assay also showed that the LEDH-FMK treatment led to increased cell viability in a dose-dependent manner compared with the cells without LEHD-FMK treatment ($P < 0.05$, Fig. 6*D*). These results further suggest that activation of apoptosis pathway in the MAD2 expressing cells is responsible for the MAD2-induced sensitization to cisplatin in CNE2 cells.

Discussion

In this study, we provide the first evidence that MAD2 expression induces chemosensitization to a DNA-damaging agent, cisplatin, which is attributed to prolonged mitotic arrest and subsequent activation of apoptosis pathway. These results further support the notion that spindle checkpoint may play an important part in DNA damage-induced cell cycle arrest. Our findings also define a novel role of MAD2 in cellular sensitivity to cisplatin.

Mitotic checkpoint acts in mitosis and delays the onset of anaphase when errors occur in the spindle structure or in the alignment of the chromosomes on the spindle (1). Ataxia telangiectasia mutated pathway has been suggested to be mainly responsible for a G_2 DNA damage checkpoint that ensures the repair of DNA damage occurred after S-phase completion (32). However, several recent studies indicate that DNA damage-induced mitotic arrest can also occur independent of DNA damage checkpoint (12–14). Although the underlying mechanisms have not been elucidated, it is understood that extensive but not minor DNA damage may lead to impairment of kinetochores leading to activation of the spindle checkpoint (12,13,33). In addition, cisplatin has been shown to directly bind to tubulin. The platinated tubulins lose their ability to assemble into microtubules, thereby blocking chromosomal segregation and activating spindle checkpoint (14). More interestingly, the same study also suggested that the number of platinated tubulins was associated with cell survival (14). In the present study, the cisplatin-induced mitotic arrest was associated with increased sensitivity in cells expressing high levels of MAD2 protein (Figs. 1-4). In addition, the cisplatin-induced mitotic arrest was more significant when high dose of cisplatin was used ($\sim$30 times of the IC_{50} dose; Fig. 3*C* and *D*). This agrees with a previous study that only extensive DNA damage, other than minor damage, induced mitotic checkpoint activation (13). These results indicate that the MAD2-induced mitotic arrest may be crucial in cisplatin-induced apoptosis in CNE2 cells.

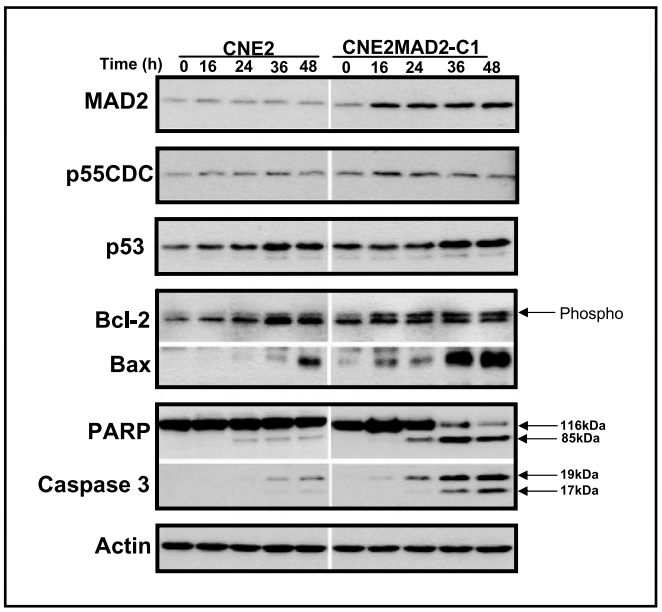


Figure 5. Western blotting analysis of apoptosis related protein expression in CNE2 cells expressing high and low levels of MAD2 protein. Cells were cultured in both ponasterone A and cisplatin (1.0 μg/mL) and protein expression was analyzed at indicated time points. Results of three independent experiments. Note the increased amount of phosphorylated Bcl-2, Bax, cleaved caspase 3, and cleaved PARP in the cells expressing higher levels of MAD2 protein.

Majority of anticancer drugs induce cytotoxicity through induction of cell cycle arrest and the prolonged cell cycle block in turn triggers apoptosis leading to cell death (24). In this study, we found that the cisplatin-induced mitotic arrest in MAD2 expressing cells was associated with decreased cell proliferation rate and increased apoptosis rate (Fig. 4). The differential cellular response between the MAD2 expressing cells and the controls was also reflected in the Western blotting analysis that increased Bcl-2 phosphorylation and Bax expression, which indicate activation of apoptosis pathway, were observed in CNE2-MAD2 cells after exposure to cisplatin (Fig. 5). In addition, activation of caspase 3 and PARP, as indicated by the appearance of cleaved fragments, also confirmed that exogenous MAD2 expression facilitated cisplatin-induced activation of apoptosis pathway in CNE2 cells. Furthermore, the evidence that activation of the apoptosis pathway by treating CNE2 cells with a Bcl-2 inhibitor, HA14-1, led to increased cell viability in response to cisplatin treatment, whereas suppression of apoptosis pathway using a caspase 9 inhibitor, LEDH, resulted in decreased cisplatin-induced cell death, further supports the hypothesis that the MAD2-induced sensitivity to cisplatin is mediated through activation of apoptosis pathway.

Another interesting point emerged from this study is that the MAD2-induced chemosensitization to cisplatin seems to be independent of p53. As shown in Fig 5, p53 protein levels were increased after cisplatin treatment in a time-dependent manner regardless of MAD2 protein levels. In addition, previous studies showed that a p53 point mutation at codon 280 was present in CNE2 cells (26). However, this point mutation is not one of the hotspots of *p53* gene mutation frequently detected in NPC (34) and its role in DNA damage response in NPC cells has not been reported. Nevertheless, the results shown in the present study indicate that the cisplatin-induced mitotic arrest in MAD2 expressing CNE2 cells may not be mediated through p53-dependent DNA damage checkpoint. These findings also agree

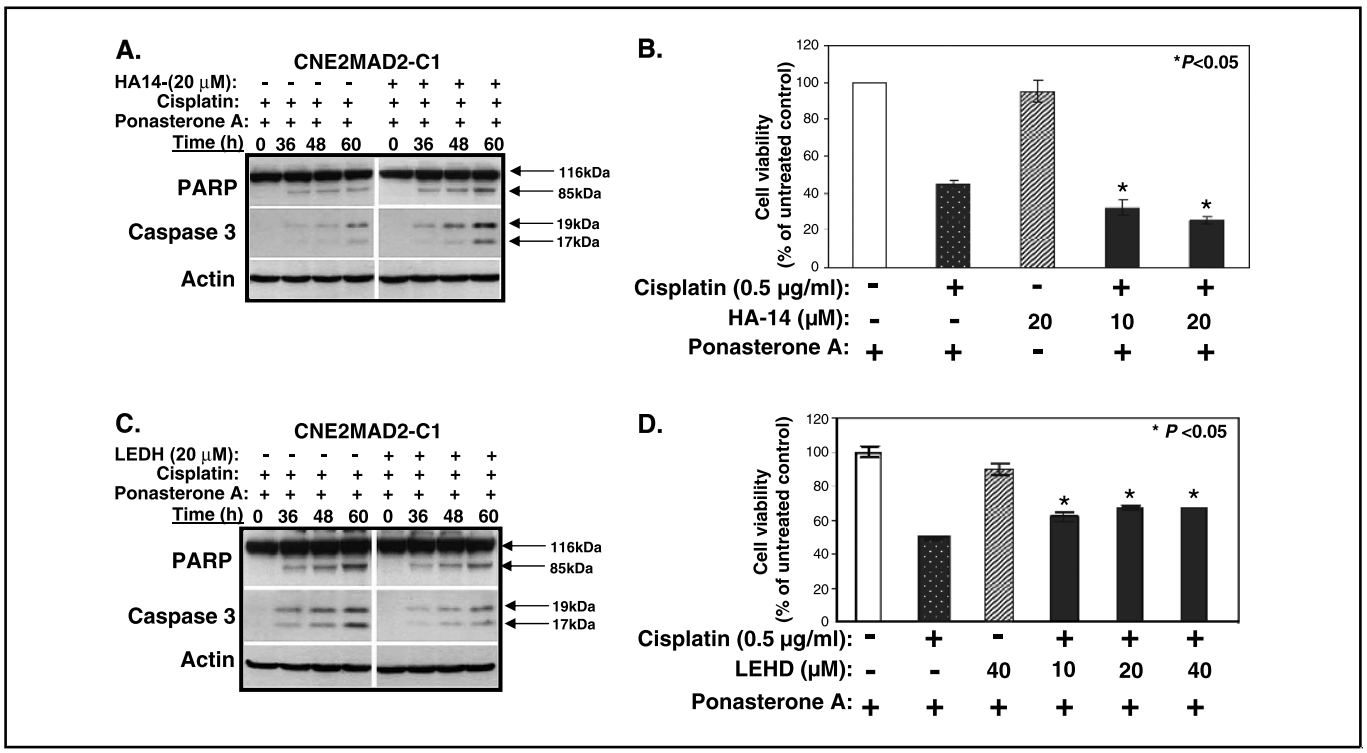


Figure 6. Effect of Bcl-2 and caspase inhibitors, HA14-1 and LEDH on the MAD2-induced sensitization to cisplatin. CNE2MAD2-C1 cells were treated with cisplatin (0.5 μg/mL) and ponasterone A with or without the inhibitors for indicated periods. Cells treated with HA14-1 (or LEDH-FMK) and ponasterone A alone was used as toxicity control in the cell viability studies. *A,* effect of HA14-1 on the expression of cleaved PARP and caspase 3. *B,* effect of HA14-1 treatment on cell viability after exposure to cisplatin by MTT assay. Note that after exposure to HA14-1, the expression of cleaved PARP and caspase 3 is increased which is associated with decreased cell viability in response to cisplatin. *C,* effect of LEDH on expression of cleaved PARP and caspase 3. *D,* effect of LEDH treatment on cell viability after exposure to cisplatin by MTT assay. Note that LEDH treatment leads to decreased expression of cleaved PARP and caspase 3 which is associated with increased cell viability in response to cisplatin.

with previous findings that spindle checkpoint itself does not require p53 (35, 36) and that DNA damage responses are independent of p53 when the mitotic checkpoint is activated (33). Recently, similar findings were reported in colon cancer cells that a functional mitotic checkpoint control is crucial in response to DNA-damaging agent–induced mitotic arrest and subsequent cell death in p53-deficient cancer cells (15). For example, cancer cells with both p53 mutations and a defective mitotic checkpoint (as a result of MAD2 inactivation through small RNA interference) are not able to undergo mitotic arrest in response to DNA damage, resulting in abnormal mitosis and subsequent replication of their damaged genomes. Although a large number of these cells will eventually die, a number of them will recover and become resistance to further drug treatment (15). These results further support our findings that decreased MAD2 expression is correlated to resistance to DNA-damaging agents. However, the molecular mechanisms responsible for the differential cell death rate between the mitotic checkpoint–competent and –defective cells, especially its relation to the apoptosis pathway were not investigated in the previous study (15). Because DNA-damaging agents such as cisplatin are commonly used anticancer drugs and resistance is the main problem of treatment failure in many cancers, especially advanced cancers with a nonfunctional p53 pathway, in combination with previous data (12–15), the present study may reveal a novel strategy that confers chemosensitization by restoration of MAD2 protein expression in human cancer cells.

Our findings in this study have implications in cancer treatment because in addition to p53 inactivation, chromosomal instability and mitotic checkpoint defects are common events in advanced human cancer. Our study suggests that down-regulation of MAD2 may play a part in the low treatment response generally observed in these cancers possibly because of its inability to induce mitotic arrest and subsequent suppression of apoptosis pathway in response to certain anticancer drugs. Because cisplatin is one of the widely used anticancer drugs, down-regulation of MAD2 could also be an indicator for identification of potential cisplatin-resistant cancers. In addition, restoration of MAD2 expression may increase the efficiency of cisplatin-based treatment. However, cells from additional cancer types need to be included in future studies to reveal if the MAD2-induced sensitization is a general phenomenon or just a specific effect in NPC cells. In addition, the molecular mechanisms responsible for decreased MAD2 expression in cancercells are required further investigation. Because deletion and mutation of the *MAD2* gene have been reported to be rare events in cancer cells (7, 37–39), we are currently studying if the hypermethylation of the *MAD2* gene promoter region is responsible for the inactivation of MAD2 in NPC cells.

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References

1. Rudner AD, Murray AW. The spindle assembly checkpoint. Curr Opin Cell Biol 1996;8:773–80.
2. Lengauer C, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. Nature 1998;396:643–9.
3. Solomon E, Borrow J, Goddard AD. Chromosome aberrations and cancer. Science 1991;254:1153–60.
4. Dobles M, Liberal V, Scott ML, Benezra R, Sorger PK. Chromosome missegregation and apoptosis in mice lacking the mitotic checkpoint protein Mad2. Cell 2000;101:635–45.
5. Michel LS, Liberal V, Chatterjee A, et al. MAD2 haploinsufficiency causes premature anaphase and chromosome instability in mammalian cells. Nature 2001;409:355–9.
6. Pennisi E. Cell division gatekeepers identified. Science 1998;279:477–8.
7. Takahashi T, Haruki N, Nomoto S, et al. Identification of frequent impairment of the mitotic checkpoint and molecular analysis of the mitotic checkpoint genes, hsMAD2 and p55CDC, in human lung cancers. Oncogene 1999;18:4295–300.
8. Li Y, Benezra R. Identification of a human mitotic checkpoint gene: hsMAD2. Science 1996;274:246–8.
9. Wang X, Jin DY, Wong YC, et al. Correlation of defective mitotic checkpoint with aberrantly reduced expression of MAD2 protein in nasopharyngeal carcinoma cells. Carcinogenesis 2000;21:2293–7.
10. Wang X, Jin DY, Ng RW, et al. Significance of MAD2 expression to mitotic checkpoint control in ovarian cancer cells. Cancer Res 2002;62:1662–8.
11. Wang X, Jin DY, Wong HL, Feng H, Wong YC, Tsao SW. MAD2-induced sensitization to vincristine is associated with mitotic arrest and Raf/Bcl-2 phosphorylation in nasopharyngeal carcinoma cells. Oncogene 2003;22:109–16.
12. Garber PM, Rine J. Overlapping roles of the spindle assembly and DNA damage checkpoints in the cell-cycle response to altered chromosomes in *Saccharomyces cerevisiae*. Genetics 2002;161:521–34.
13. Mikhailov A, Cole RW, Rieder CL. DNA damage during mitosis in human cells delays the metaphase/anaphase transition via the spindle-assembly checkpoint. Curr Biol 2002;12:1797–806.
14. Tulub AA, Stefanov VE. Cisplatin stops tubulin assembly into microtubules. A new insight into the mechanism of antitumor activity of platinum complexes. Int J Biol Macromol 2001;28:191–8.
15. Nitta M, Kobayashi O, Honda S, et al. Spindle checkpoint function is required for mitotic catastrophe induced by DNA-damaging agents. Oncogene 2004;23:6548–58.
16. Zhang S, Wu Y, Zeng Y, Zech L, Klein G. Cytogenetic studies on an epithelioid cell line derived from nasopharyngeal carcinoma. Hereditas 1982;97:23–8.
17. Gu SY, Tang WP, Zeng Y, Zhao EW, Deng WH, Li K. An epithelial cell line established from poorly differentiated nasopharyngeal carcinoma. Chinese Journal of Cancer 1983;2:270–2.
18. Glaser R, Zhang HY, Yao KT, et al. Two epithelial tumor cell lines (HNE-1 and HONE-1) latently infected with Epstein-Barr virus that were derived from nasopharyngeal carcinomas. Proc Natl Acad Sci U S A 1989;86:9524–8.
19. Huang DP, Ho JH, Poon YF, et al. Establishment of a cell line (NPC/HK1) from a differentiated squamous carcinoma of the nasopharynx. Int J Cancer 1980;26:127–32.
20. Lin CT, Wong CI, Chan WY, et al. Establishment and characterization of two nasopharyngeal carcinoma cell lines. Lab Invest 1990;62:713–24.
21. Tsao SW, Wang X, Liu Y, et al. Establishment of two immortalized nasopharyngeal epithelial cell lines using SV40 large T and HPV16E6/E7 viral oncogenes. Biochim Biophys Acta 2002;1590:150–8.
22. Ling MT, Wang X, Ouyang XS, Xu K, Tsao SW, Wong YC. Id-1 expression promotes cell survival through activation of NF-κB signalling pathway in prostate cancer cells. Oncogene 2003;22:4498–508.
23. Wang X, Wong SC, Pan J, et al. Evidence of cisplatin-induced senescent-like growth arrest in nasopharyngeal carcinoma cells. Cancer Res 1998;58:5019–22.
24. Hochhauser D. Modulation of chemosensitivity through altered expression of cell cycle regulatory genes in cancer. Anticancer Drugs 1997;8:903–10.
25. Lin M, Chang JK, Shankar D, Sakamoto KM. The role of p55CDC in cell cycle control and mammalian cell proliferation, differentiation, and apoptosis. Exp Mol Pathol 2003;74:123–8.
26. Spruck CH III, Tsai YC, Huang DP, et al. Absence of p53 gene mutations in primary nasopharyngeal carcinomas. Cancer Res 1992;52:4787–90.
27. Blagosklonny MV, Giannakakou P, el-Deiry WS, et al. Raf-1/bcl-2 phosphorylation: a step from microtubule damage to cell death. Cancer Res 1997;7:130–5.
28. Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE, Poirier GG. Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. Cancer Res 1993;53:3976–85.
29. Stennicke HR, Salvesen GS. Biochemical characteristics of caspases-3, -6, -7, and -8. J Biol Chem 1997;272:25719–23.
30. Wang JL, Liu D, Zhang ZJ, et al. Structure-based discovery of an organic compound that binds Bcl-2 protein and induces apoptosis of tumor cells. Proc Natl Acad Sci U S A 2000;97:7124–9.
31. Yamaguchi H, Paranawithana SR, Lee MW, Huang Z, Bhalla KN, Wang HG. Epothilone B analogue (BMS-247550)-mediated cytotoxicity through induction of Bax conformational change in human breast cancer cells. Cancer Res 2002;62:466–71.
32. Shiloh Y. ATM and ATR: networking cellular responses to DNA damage. Curr Opin Genet Dev 2001;11:71–7.
33. Chow JP, Siu WY, Fung TK, et al. DNA damage during the spindle-assembly checkpoint degrades CDC25A, inhibits cyclin-CDC2 complexes, and reverses cells to interphase. Mol Biol Cell 2003;14:3989–4002.
34. Van Tornout JM, Spruck CH III, Shibata A, et al. Presence of p53 mutations in primary nasopharyngeal carcinoma (NPC) in non-Asians of Los Angeles, California, a low-risk population for NPC. Cancer Epidemiol Biomarkers Prev 1997;6:493–7.
35. Di Leonardo A, Khan SH, Linke SP, Greco V, Seidita G, Wahl GM. DNA rereplication in the presence of mitotic spindle inhibitors in human and mouse fibroblasts lacking either p53 or pRb function. Cancer Res 1997;57:1013–9.
36. Minn AJ, Boise LH, Thompson CB. Expression of Bcl-xL and loss of p53 can cooperate to overcome a cell cycle checkpoint induced by mitotic spindle damage. Genes Dev 1996;10:2621–31.
37. Gemma A, Hosoya Y, Seike M, et al. Genomic structure of the human MAD2 gene and mutation analysis in human lung and breast cancers. Lung Cancer. 2001;32:289–95.
38. Hernando E, Orlow I, Liberal V, Nohales G, Benezra R, Cordon-Cardo C. Molecular analyses of the mitotic checkpoint components hsMAD2, hBUB1 and hBUB3 in human cancer. Int J Cancer 2001;95:223–7.
39. Percy MJ, Myrie KA, Neeley CK, Azim JN, Ethier SP, Petty EM. Expression and mutational analyses of the human MAD2L1 gene in breast cancer cells. Genes Chromosomes Cancer 2000;29:356–62.

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