ORIGINAL RESEARCH

TopBP1 contributes to the chemoresistance in non-small cell lung cancer through upregulation of p53

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Abstract: Resistance to chemotherapeutic drugs is a major obstacle in non-small cell lung cancer (NSCLC) therapy. The molecular determinants of NSCLC resistance to doxorubicin are unknown. In the present study, we investigated whether topoisomerase IIβ binding protein 1 (TopBP1) was involved in the chemoresistance to doxorubicin in NSCLC cancer. We found that p53-deficient lung cancer cells (NCI-H1299) displayed the greatest resistance to doxorubicin compared with NCI-H358, A549, and HCC827 cells with p53 expression. The expression of TopBP1 was significantly higher in NCI-H1299 cells than the other three tumor cell lines. In addition, TopBP1 knockdown with specific small interfering RNA in NCI-H1299 cells enhanced the doxorubicin chemosensitivity and decreased the expression of p53 in the presence of doxorubicin. After doxorubicin administration, co-immunoprecipitation assay showed that TopBP1 promoted the expression of p53 in NCI-H1299 cells. These results for the first time demonstrated that TopBP1 plays an important role in NSCLC chemoresistance via upregulation of p53. Therefore, inhibition of TopBP1, in combination with chemotherapy, may represent a novel strategy for the treatment of chemotherapy-resistant NSCLC.

Keywords: non-small cell lung cancer, drug resistance, TopBP1, p53

Introduction

Lung cancer is the leading cause of cancer-related death worldwide; 80% of lung cancers are non-small cell lung cancer (NSCLC) with poor therapeutic efficacy when diagnosed.[1,2] It is estimated that approximately 40% of patients with NSCLC present with advanced-stage disease for which 5-year survival rates are in the region of 2%.[3] Currently, platinum-based regimens are the mainstay of lung cancer therapy, and chemotherapy serves as one of the important adjuvant therapies for its treatment.[4] However, drug resistance to conventional chemotherapeutics has become a major handicap in the success of NSCLC chemotherapy.[5,6] Thus, it is imperative to develop novel therapeutic strategies that may enhance tumor cell response to anticancer drugs. Recently, studies have begun to investigate the molecular mechanism of NSCLC and identified various novel targeted agents, such as epidermal growth factor receptor tyrosine kinase inhibitors which exhibit greater efficacy than chemotherapy in patients with epidermal growth factor receptor-mutated tumors.[7] Despite the great progresses achieved in cancer therapy, the molecular mechanism of lung cancer pathogenesis and chemoresistance still remains elusive.

Topoisomerase IIβ binding protein 1 (TopBP1) was identified as an interacting partner for topoisomerase IIβ.[8,9] It contains nine BRCA1 carboxyl-terminal domains and functions in DNA damage response, DNA checkpoint activation, replication, and

regulation of transcription.[10–13] TopBP1 also interacts several transcriptional factors, such as p53,[14] E2F1,[15,16] Miz1,[17] and 53BP1.[18] Regulation of p53 by TopBP1 plays an important role in the regulation of proapoptotic activity and cell cycle transition.[19] It is reported that TopBP1 is frequently overexpressed in cancer and inactivates p53 functions.[19] In the current study, we aimed to explore the biological role of TopBP1 in chemoresistance along with the molecular mechanism underling these effects.

Materials and methods
Cell culture and reagents
Human lung cancer cell lines NCI-H358, A549, NCI-H1299, and HCC827 were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were maintained at 37°C in 5% CO_2 incubator. Doxorubicin was purchased from Sigma-Aldrich (St Louis, MO, USA). The TopBP1 small interfering RNA (siRNA) and negative control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). As clinical samples or animals were not used in this study ethical approval was not required by the institutional review board.

CCK-8 assay
Cancer cells or siRNA-transfected cancer cells were seeded onto 96-well plates at 3,000 cells/well. The medium was replaced with the corresponding serum-free medium for 24 hours to synchronize the cell cycle, and then the serum-free medium was replaced with complete medium containing the drugs at the indicated concentrations. Then, 10 μL/well CCK-8 solution (Dojindo, Kumamoto, Japan) was added, the plates incubated for 3 hours, and absorbance was measured at 450 nm using an MRX II microplate reader (Dynex, Chantilly, VA, USA).

EdU incorporation assay
Cell proliferation was calculated using EdU incorporation assay. Measurement of the inhibitive rate of cell proliferation was carried out using a Click-iTEdU Imaging Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacture's protocol.

Flow cytometry analysis
Tumor cells were exposed to doxorubicin and p53 siRNA alone or in combination. After treatment for 48 hours, cells were trypsinized and centrifuged rpm for 5 minutes and the pellet washed twice with phosphate-buffered saline (PBS).

Cells were resuspended and then washed with PBS three times. Apoptosis cells were detected with annexin V-FITC/PI according to the protocol of Annexin V-FITC cell Apoptosis Detection Kit (Sigma-Aldrich Co., St Louis, MO, USA).

siRNA transfection
Lung cancer cells were seeded to achieve 30%–50% confluency on the day of transfection. Cells were transfected with TopBP1 siRNA, p53 siRNA, or negative control siRNA using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instruction. The transfection medium (Opti-MEM; Gibco) was replaced with complete medium 12 hours after transfection for further analysis.

Western blot analysis
Cells were lysed in 50 μL cell lysis buffer (Cell Signaling, Danvers, MA, USA). The protein concentration was quantified using the BCA Protein Kit (Thermo, Rockford, IL, USA). Cell lysates were separated by 10% SDS-PAGE and proteins were transferred to polyvinylidenedifluoride membranes (Merck Millipore, Billerica, MA, USA). The membranes were then incubated with anti-TopBP1 or anti-p53 antibodies (Abcam, Cambridge, MA, USA) at 4°C overnight. The membranes were washed three times with TBS/T and then incubated with the appropriate HRP-conjugated secondary antibodies (Abcam) for 1 hour at room temperature. Protein expression was detected by chemiluminescence (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA).

Co-immunoprecipitation
Cells were harvested and lysed in 50 μL cell lysis buffer (Cell Signaling) after treatment with doxorubicin for 48 hours. An aliquot of the cell lysates was lysed with SDS lysis buffer, and the rest of the cell lysates were incubated with appropriate antibodies or beads for 24 hours at 4°C. Immunoprecipitates were fractionated by SDS-PAGE and electrotransferred to the polyvinylidenedifluoride membranes (Merck Millipore). The specific signals were detected with anti-TopBP1 or anti-p53 antibodies (Abcam). Protein expression was detected using chemiluminescence (GE Healthcare Bio-Sciences Corp.).

Statistical analysis
Each experiment was performed in triplicate and repeated at least three times. All the data were presented as mean ± standard deviation and treated for statistical analysis by Statistical Product and Service Solutions (SPSS) 16.0 (IBM, Armonk, NY, USA). Comparison between groups was made using one-way ANOVA and a statistically significant difference was defined as $P<0.05$.

Results
Different doxorubicin sensitivity and TopBP1 expression in lung cancer cells

Firstly, CCK-8 assay we performed to examine cell viability of different lung cancer cell lines (NCI-H358, A549, NCI-H1299, and HCC827) after incubation with doxorubicin for 48 and 72 hours. It was found that the doxorubicin sensitivity varied among cell lines (Figure 1A–D). As shown in Table 1, the

IC_{50} values at 48 or 72 hours were significantly higher in NCI-H1299 cells compared with the other three cell lines. In addition, the protein expression of TopBP1 was detected in different cell lines by Western blot. We found that TopBP1 expression was significantly higher in NCI-H1299 cells compared with three other cell lines (Figure 1E). Furthermore, EdU incorporation assay was carried out to measure the proliferation of lung cancer cells. Results showed that the inhibitive effect of
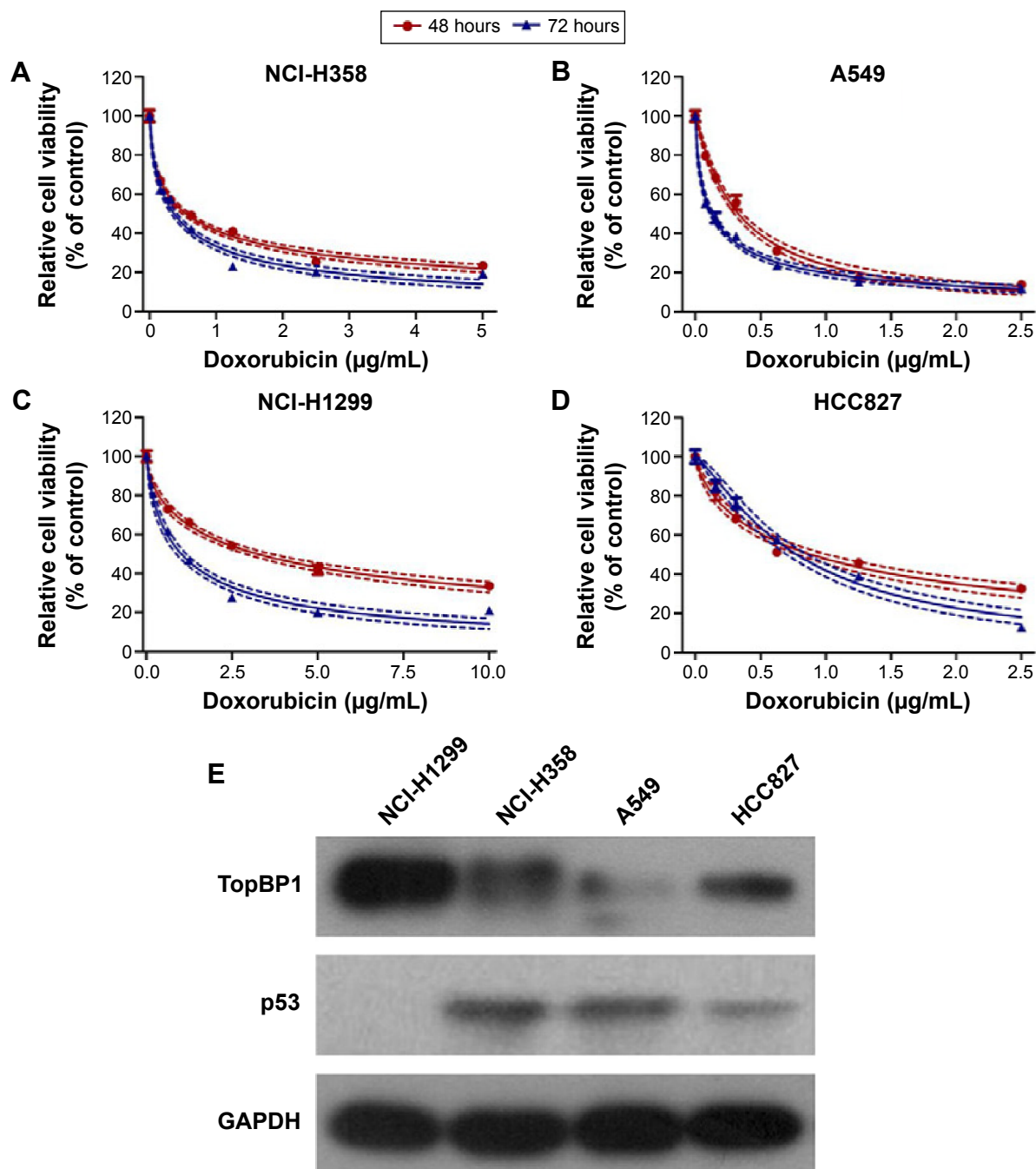
Figure 1 Different doxorubicin sensitivity and topoisomerase IIβ binding protein 1 (TopBP1) expression in lung cancer cells.
Notes: Lung cancer cells were incubated with different concentrations of doxorubicin for 48 or 72 hours. CCK-8 assay was applied to measure the cell viability of different lung cancer including NCI-H358 (**A**), A549 (**B**), NCI-H1299 (**C**), and HCC827 (**D**). Western blot was used to detect the protein expression of TopBP1 in four cancer cell lines (**E**).

Table 1 Determination of doxorubicin IC$_{50}$ in different tumor cell lines

IC$_{50}$ (μg/mL)	48 hours	72 hours
NCI-H358	0.5409	0.3677
A549	0.3304	0.1263
HCC827	0.8896	0.777
NCI-H1299	3.423	1.030

Abbreviation: IC$_{50}$, 50% inhibitory concentration.

doxorubicin was reduced in NCI-H1299 cells compared with that in NCI-H358, A549, and HCC827 cells (Figure 2A–D). These results implied that the TopBP1 might be associated with the drug resistance to doxorubicin in NSCLC cells.

TopBP1 was involved in the chemoresistance of NSCLC cells

In order to confirm whether TopBP1 was involved in the tumor cells chemoresistance, TopBP1 siRNA was transfected into lung cancer cells to interfere with TopBP1 expression. Western blot showed that the TopBP1 expression was obviously decreased in four cancer cell lines after transfection with TopBP1 siRNA (Figure 3A). Addition of doxorubicin increased the p53 expression in NCI-H358, A549, and HCC827 cells and, interestingly, induced the expression of p53 in NCI-H1299 cells (Figure 3A). Moreover, we found
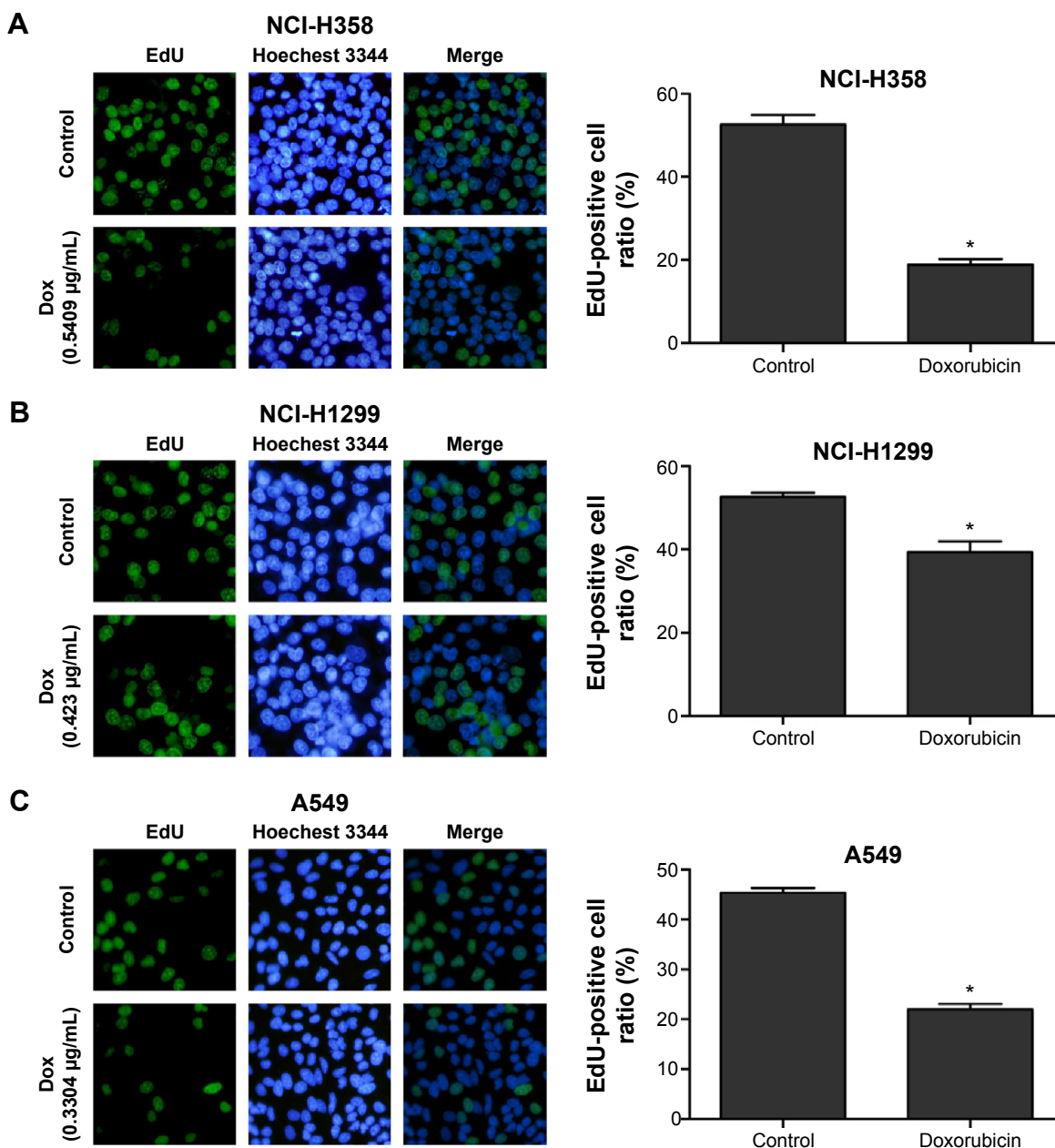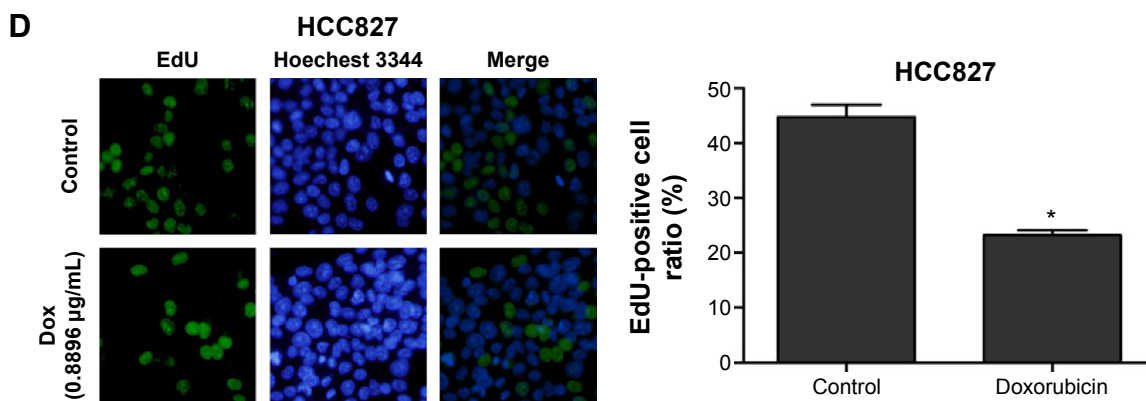

Figure 2 (*Continued*)

Figure 2 Measurement of cell proliferation in lung cancer cells.
Notes: Lung cancer cells were incubated with or without doxorubicin (Dox) for 48 hours. EdU incorporation assay was performed to measure the proliferation of NCI-H358 (**A**), NCI-H1299 (**B**), A549 (**C**), and HCC827 (**D**) cells. *$P<0.05$.

that p53 siRNA-transfected NCI-H1299 cells became more sensitive to doxorubicin, while downregulation of p53 in other three cell lines led to no significant changes in their cellular responses to doxorubicin treatment (Figure S1). CCK-8 assay demonstrated that TopBP1 knockdown increased the sensitivity of NCI-H1299 (Figure 3B), NCI-H358, A549, and HCC827 cells (Figure S2) to doxorubicin. These data suggested that TopBP1 was involved in NSCLC cell sensitivity to doxorubicin.

Downregulation of TopBP1 sensitized NCI-H1299 cells to doxorubicin

We further performed EdU incorporation assay to measure the proliferation of lung cancer cells after transfection with TopBP1 siRNA. Results showed that TopBP1 downregulation led to no significant differences in the proliferation of NCI-H358 (Figure 4A), A549 (Figure 4C), and HCC827 (Figure 4D) cells in the presence of doxorubicin. However, the inhibitive effect of doxorubicin was increased in
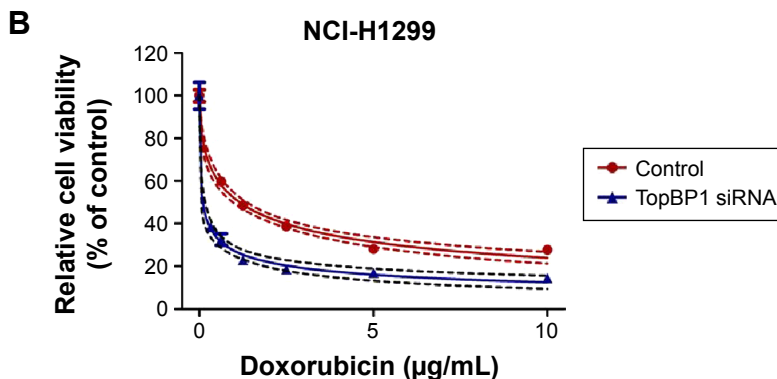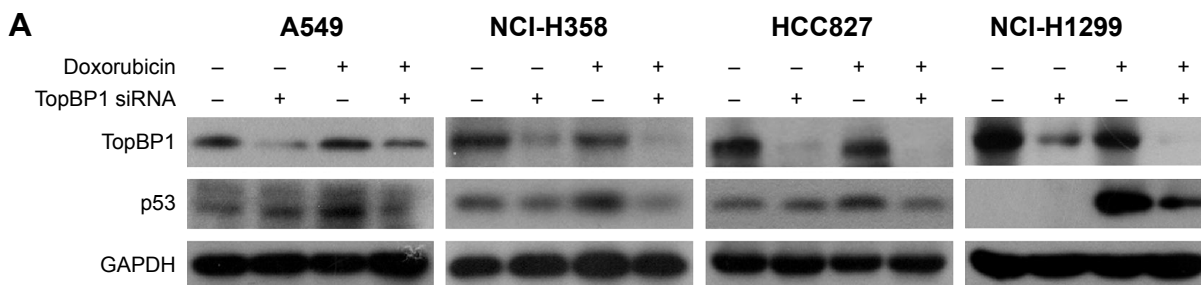

Figure 3 TopBP1 was involved in the chemoresistance of tumor cells.
Notes: TopBP1 siRNA was transfected into different lung cancer cells and Western blot was applied to detect the protein levels of TopBP1 and p53 in tumor cells with or without doxorubicin treatment (**A**). After transfection, the chemosensitivity of NCI-H1299 cells to doxorubicin was measured using CCK-8 methods (**B**).
Abbreviations: TopBP1, topoisomerase IIβ binding protein 1; siRNA, small interfering RNA.
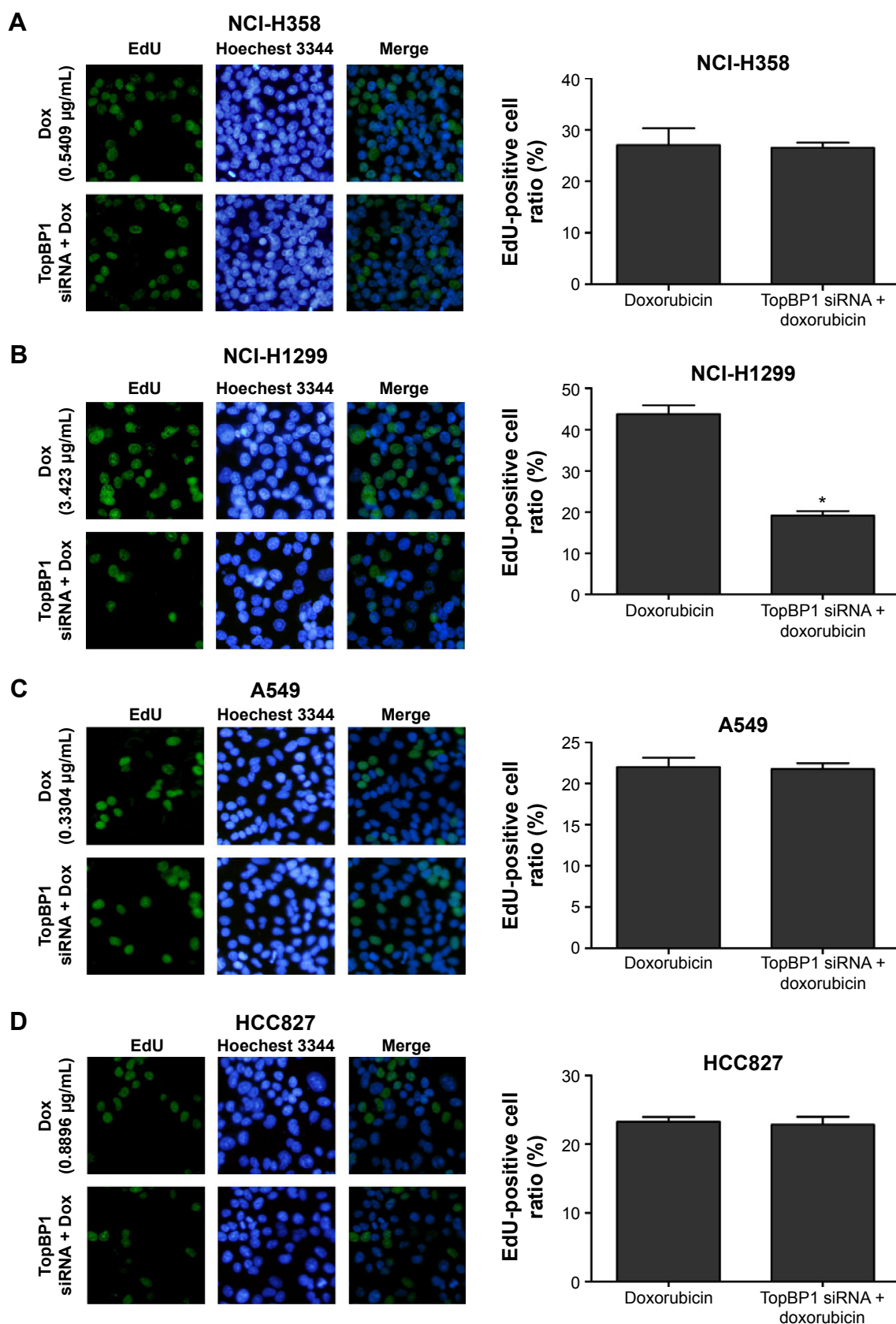
Figure 4 Measurement of cell proliferation in TopBP1 siRNA-transfected lung cancer cells.
Notes: Lung cancer cells were transfected with TopBP1 siRNA or control siRNA in the presence of doxorubicin (Dox). EdU incorporation assay was performed to measure the proliferation of NCI-H358 (**A**), NCI-H1299 (**B**), A549 (**C**), and HCC827 (**D**) cells. *$P<0.05$.
Abbreviations: TopBP1, topoisomerase IIβ binding protein 1; siRNA, small interfering RNA.

TopBP1 siRNA-transfected NCI-H1299 cells compared with doxorubicin treatment alone (Figure 4B).

Doxorubicin-induced p53 was mediated by TopBP1 in NSCLC cells

Finally, co-immunoprecipitation was performed to investigate the relationship between TopBP1 and doxorubicin-induced p53. It was demonstrated that TopBP1 was involved in the p53 induction after doxorubicin administration in NCI-H1299 cells (Figure 5A). However, there are little changes in p53 levels in NCI-H358, A549, and HCC827 cells (Figure 5B). These results showed that the drug resistance was regulated by TopBP1-dependent p53 induction by doxorubicin in NCI-H1299 cells.

Discussion

Currently, chemotherapy serves as an important component of postoperative treatment for NSCLC. However, development of drug resistance to the conventional chemotherapeutics has become a major challenge to successful chemotherapy.[20,21] Thus, it is urgent that we find novel therapeutic targets involved in the acquisition of drug resistance. In this study, we demonstrated that TopBP1 modulated the chemoresistance to doxorubicin in NSCLC cells. It is noteworthy to mention that, to our knowledge, this study was the first to demonstrate the therapeutic potential of TopBP1 in lung cancer.

TopBP1 has been shown to be involved in DNA damage response, chromosome replication, and transcription.[10–13] Depletion of TopBP1 led to embryonic lethality at the peri-implantation stage and cellular senescence in primary cells.[22,23] In breast cancer, Liu et al observed overexpression of TopBP1 in tumor tissues and demonstrated that those patients with higher expression of TopBP1 had shorter overall survival time than those without overexpression of TopBP1.[14] In a recent study, the expression of TopBP1 was found to be upregulated in cancer cells and depletion of TopBP1 enhanced the sensitivity to radiation in radio-resistant lung cancer cells.[24] Our study found that NCI-H1299 cells exhibited the lowest sensitivity to doxorubicin among four lung cancer cell lines. Interestingly, Western blot analysis showed that the protein level of TopBP1 was higher in NCI-H1299 cells compared with NCI-H358, A549, and HCC827 cells. Thus, it was hypothesized that enhanced expression of TopBP1 might be involved in the chemoresistance to doxorubicin. To test such a hypothesis, the TopBP1 expression was decreased via transfection of TopBP1 siRNA into NCI-H1299 cells. Consequently, TopBP1 knockdown in
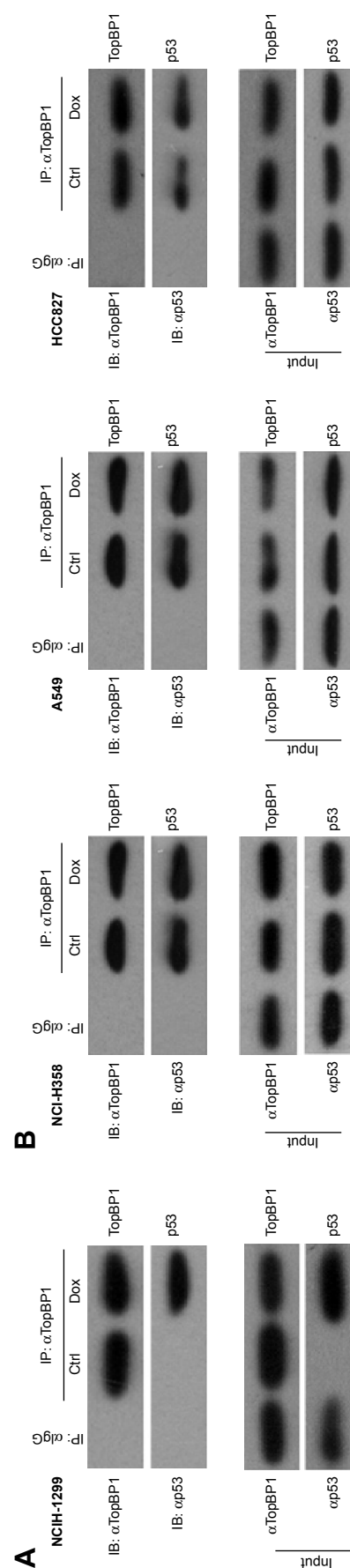

Figure 5 Upregulation of p53 by topoisomerase IIβ binding protein 1 (TopBP1) in NCI-H1299 cells.
Notes: Lung cancer cells were incubated with or without doxorubicin for 48 hours. Co-immunoprecipitation assay was applied to detect the expression of TopBP1 and p53 in NCI-H1299 (**A**), NCI-H358, A549, and HCC827 cells (**B**).
Abbreviations: Ctrl, control; Dox, doxorubicin; TopBP1, topoisomerase IIβ binding protein 1.

NCI-H1299 cells increased the sensitivity to doxorubicin. These results suggested that TopBP1 was a critical modulator in the acquired chemoresistance of lung cancer cells.

The tumor suppressor p53 is a transcription factor that responds to various types of cellular stress, such as oncogene activation and genotoxic drug-induced DNA damage.[25,26] p53 regulates a variety of cellular functions, including DNA repair, cell cycle arrest, and apoptosis.[27–29] Abnormal expression of p53 is found in nearly all types of cancers, and p53 mutations are associated with resistance to chemotherapeutics and poor patient prognosis.[30–33] In our study, the p53-negative NCI-H1299 cells displayed more doxorubicin resistance than three other cell lines with p53 expression. In order to explore the role of p53 in TopBP1-modulated chemosensitivity, we examined TopBP1 and p53 expression with or without doxorubicin treatment. Our data showed that doxorubicin administration resulted in upregulation of p53 in NCI-H358, A549, and HCC827 cells. Interestingly, we found that the addition of doxorubicin led to p53 induction in NCI-H1299 cells. These results implied that, contrary to the tumor suppressive effect of p53 originally expressed in cells, doxorubicin-induced p53 probably contributed to drug resistance in lung cancer cells. In addition, co-immunoprecipitation assay showed that TopBP1 mediated the doxorubicin-induced p53 expression in NCI-H1299 cells, but not in NCI-H358, A549, and HCC827 cells.

Conclusion

In conclusion, our study for the first time demonstrated that TopBP1 is a target whose inhibition restores the sensitivity to doxorubicin in p53-null tumor cells. Furthermore, TopBP1 contributes to the drug resistance though regulation of doxorubicin-induced p53. Therefore, targeting TopBP1, in combination with chemotherapy, may represent a novel strategy for the treatment of chemotherapy-resistant tumors.

Acknowledgment

Disclosure

The authors report no conflicts of interest in this work.

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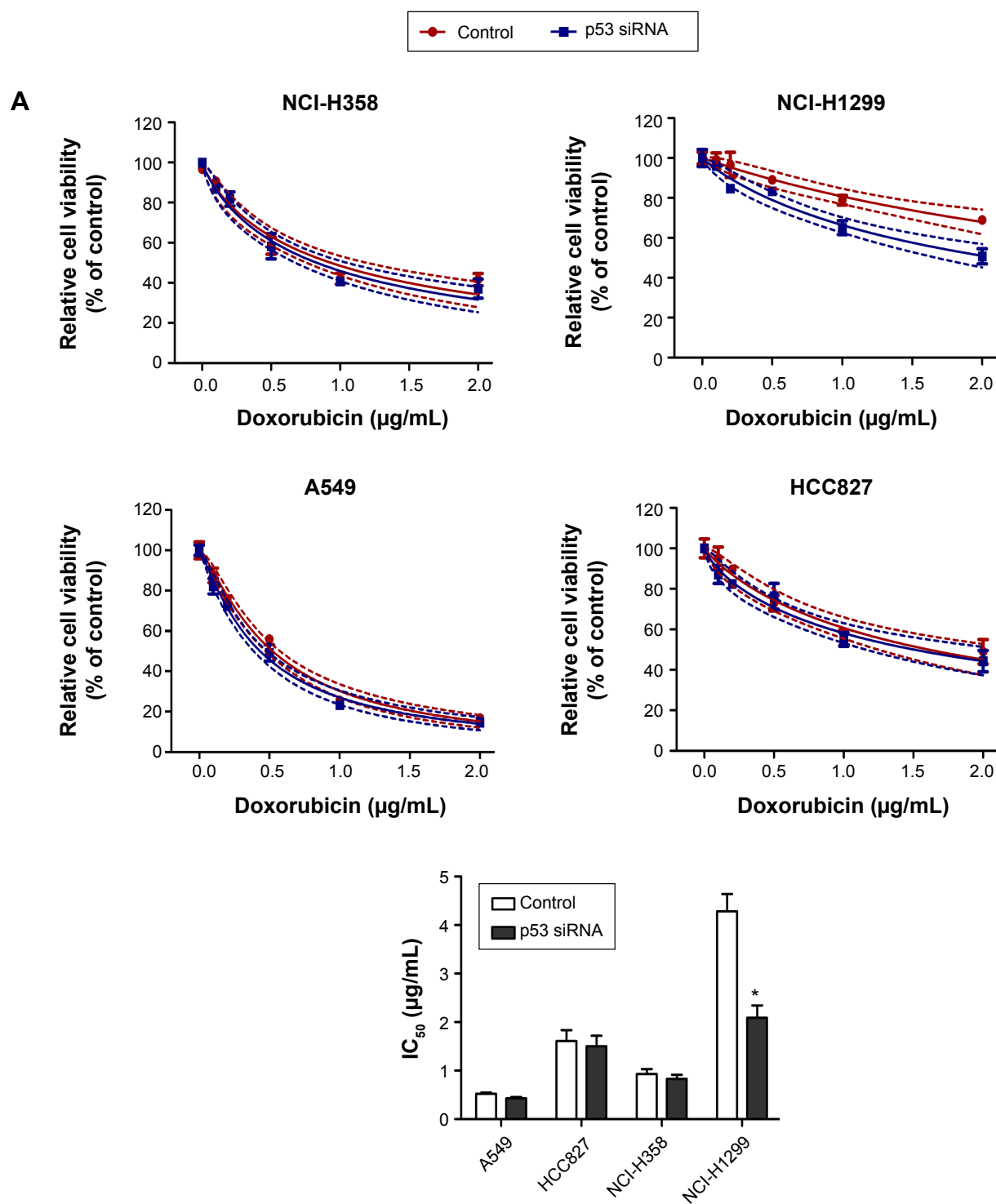
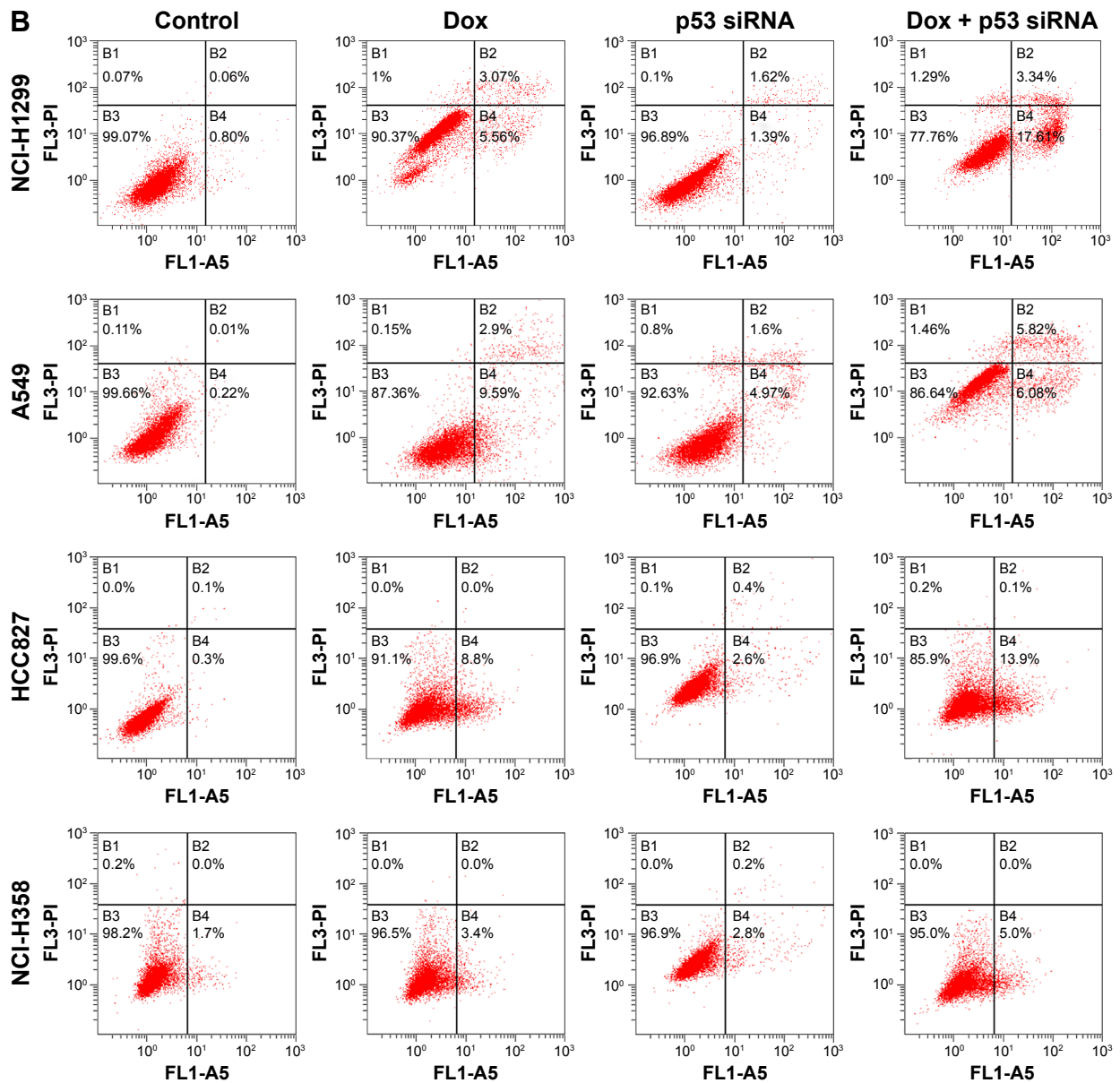# Supplementary materials

Figure S1 (*Continued*)

Figure S1 Cell viability (**A**) and apoptosis (**B**) in doxorubicin (Dox)-treated non-small cell lung cancer cells before and after p53 knockdown. * represents statistical significance with $P < 0.05$.
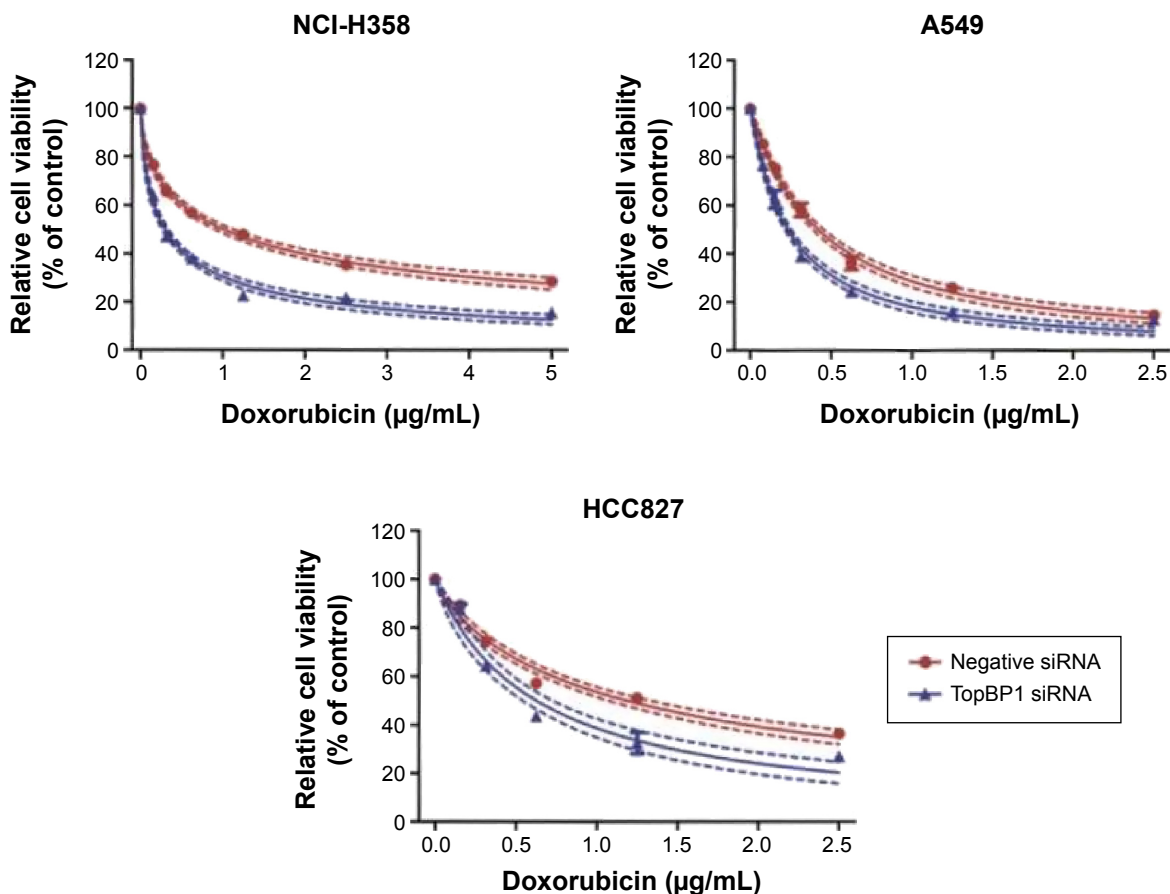**Abbreviations:** siRNA, small interfering RNA; IC$_{50}$, 50% inhibitory concentration.

NCI-H358

A549

HCC827

Negative siRNA
TopBP1 siRNA

Figure S2 Effect of TopBP1 in non-small cell lung cancer cells.
Abbreviations: TopBP1, topoisomerase IIβ binding protein 1; siRNA, small interfering RNA.