

Aurora B confers cancer cell resistance to TRAIL-induced apoptosis via phosphorylation of survivin

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Tumor necrosis factor-related apoptosis-induced ligand (TRAIL) induces apoptosis selectively in cancer cells while sparing normal cells. However, many cancer cells are resistant to TRAIL-induced cell death. In this study, we examined whether Aurora B, which is frequently overexpressed in cancer cells, is associated with TRAIL resistance. The protein levels of Aurora B were higher in TRAIL-resistant cancer cell lines than in TRAIL-sensitive cancer cell lines. Exogenously expressed Aurora B attenuated TRAIL-induced apoptosis in the tested TRAIL-sensitive cancer cell lines, whereas the small interfering RNA-mediated suppression of Aurora B expression stimulated TRAIL-mediated apoptosis in the tested TRAIL-resistant cancer cell lines. Furthermore, combined treatment with TRAIL and ZM447439, a specific inhibitor of Aurora B, synergistically induced apoptosis in various TRAIL-resistant cancer cells, suggesting that this combined regimen may represent an attractive strategy for effectively treating TRAIL-resistant malignant cancers. Mechanistically, the inhibition of Aurora B activity in various cancer cells commonly down-regulated survivin protein levels and potentiated the activation of caspase-3. In addition, Aurora B inhibition induced mitotic catastrophe, which also contributed to the sensitization of cells to TRAIL-mediated apoptosis. Interestingly, forced overexpression of Aurora B increased the protein levels of survivin, but not those of a non-phosphorylatable survivin mutant in which threonine 117 was replaced by alanine, indicating that phosphorylation of survivin is required for this effect. Furthermore, TRAIL-induced apoptosis in MDA-MB-435S cells was attenuated by wild-type survivin but not by the non-phosphorylatable survivin mutant. Collectively, our results demonstrate that Aurora B confers TRAIL resistance to cancer cells via phosphorylation of survivin.

Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is considered a potential agent for cancer therapeutics due to its ability to induce apoptosis in a variety of cancer cells without affecting the majority of normal human cells (1). However, recent studies have shown that many types of cancer cells are resistant to the apoptotic effects of TRAIL (2), suggesting that TRAIL alone may not be sufficient to treat all malignant tumors. To facilitate the future development of more effective TRAIL-based cancer therapies, we must seek to identify the

Abbreviations: cDNA, complementary DNA; HA, hemagglutinin; mRNA, messenger RNA; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; siRNA, small interfering RNA; TRAIL, tumor necrosis factor-related apoptosis-induced ligand; WT, wild-type.

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factors that contribute to TRAIL resistance in cancer cells and develop sensitizers capable of effectively restoring TRAIL sensitivity.

Members of the Aurora family of serine/threonine kinases play important roles in chromosomal alignment, segregation and cytokinesis during mitosis (3). For example, Aurora A functions in the regulation of mitotic entry, chromosomal maturation and mitotic spindle assembly (4). Aurora B is one of the chromosomal passenger proteins involved in chromosomal segregation, spindle checkpoint and cytokinesis (4,5). Aurora C is specifically expressed in the testis and plays a role in spermatogenesis (6). Elevated expression of Aurora A and Aurora B has been detected in a high percentage of breast, lung, prostate, brain and thyroid tumors (7) and their overexpression has been correlated with cancer susceptibility and poor prognosis in several types of cancers (8–12). Interestingly, expression of Aurora B often parallels that of Aurora A in normal tissues and cancer cells (13). Because of their involvements in mitotic control, genomic instability and tumorigenesis, the Aurora kinases have received considerable attention as potential targets in cancer therapy (7,14). Furthermore, recent studies have shown that Aurora A overexpression in cancer cells is closely associated with resistance to chemotherapy (15–17), suggesting that Aurora A may play an anti-apoptotic role.

Here, we show for the first time that Aurora B contributes more than Aurora A to TRAIL resistance in cancer cells. We found that the phosphorylation and subsequent increase in the protein levels of survivin by Aurora B may help cancer cells resist TRAIL-mediated apoptosis. Furthermore, we report that inhibition of Aurora B kinase activity can recover TRAIL sensitivity in TRAIL-resistant cancer cell lines via survivin downregulation. These findings suggest that a combined regimen with TRAIL and a specific inhibitor of Aurora B could offer an effective therapeutic strategy for sensitizing TRAIL-resistant cancer cells to TRAIL-mediated apoptosis.

Materials and methods

Reagents

Recombinant human TRAIL/Apo2 ligand (the non-tagged 19 kDa protein, amino acids 114–281) was from KOMA Biotech (Seoul, South Korea). 4',6-Diamidino-2-phenylindole, calcein-acetoxymethyl ester (calcein-AM) and ethidium homodimer-1 were from Molecular Probes (Eugene, OR). ZM447439 was from Biomol (Plymouth Meeting, PA). We used antibodies against Aurora A (BD Biosciences Pharmingen, San Jose, CA); Aurora B (Abcam, Cambridge, MA); DR5 (KOMA); phosphorylated histone H3 (Upstate, Lake Placid, NY); PARP (Poly (ADP ribose) polymerase) (Biomol); HA (hemagglutinin) (Covance, Berkeley, CA); Flag (Sigma, St Louis, MO); caspase-8, caspase-3, survivin and XIAP (Stressgen, Ann Arbor, MI); Bcl-2, Bcl-xL, caspase-9 and c-IAP2 (Santa Cruz Biotechnology, Santa Cruz, CA); cleaved caspase-3 and histone H3 (Cell Signaling, Beverly, MA); α -tubulin (Oncogene Science, Cambridge, MA) and rabbit IgG horseradish peroxidase, mouse IgG and goat IgG (Zymed Laboratories, South San Francisco, CA).

Culture of various cancer cells

The Hep3B and HepG2 human hepatoma cell lines, the U251MG glioma cell line, the MDA-MB-435S and Hs578T breast cancer cell lines and the SW-837, RKO, HCT116 and HT-29 colon cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). The Huh-7 and SNU-201 human hepatoma cell lines were from the Korean Cell Line Bank (Seoul, Korea). The U251N human glioma cell line, which is a subclone of the U251MG cell line (18), and the U343 glioma cell line were generously provided by Dr Yun C.O. (Hanyang University, Korea) (19,20). U251N cells were previously reported to be sensitive to TRAIL (21) and able to produce tumors in athymic nude mice (22). These various cancer cell lines were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics (Life Technologies, Grand Island, NY).

Measurement of cellular viability

After treatments, cell viability was assessed by double labeling of cells with 2 μ M calcein-AM and 4 μ M ethidium homodimer-1. The calcein-positive live

cells and ethidium homodimer-1-positive dead cells were visualized using fluorescence microscope (Axiovert 200M, Carl Zeiss, Germany) and counted.

Expression vectors encoding HA-tagged aurora A, HA-tagged aurora B, wild-type survivin or T117A mutant survivin

The full-length complementary DNA (cDNA) sequences containing the entire open reading frame of the human *Aurora A* and *Aurora B* genes were polymerase chain reaction amplified using oligo-dT primers. The resulting cDNAs for the *Aurora A* and *Aurora B* gene were subcloned into the HA epitope-encoding pcDNA3.1 vector to generate vectors encoding HA-tagged Aurora A and Aurora B proteins (pHA-Aurora A and pHA-Aurora B). A cDNA fragment containing the entire open reading frame of survivin was reverse transcription-polymerase chain reaction (RT-PCR) amplified using RNA obtained from 293T cells. This full-length cDNA fragment was inserted into the Flag epitope-encoding pcDNA3.1 vector to generate the expression vector encoding Flag-tagged survivin protein [wild-type (WT) Survivin]. For mutagenesis, WT Survivin was changed to T117A Survivin by using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

Transfection

Cells were transiently transfected using the Lipofectamine Plus™ reagent (Invitrogen, Carlsbad, CA). Cells were plated in six-well plates and grown to 70–80% confluency in antibiotic-free medium, and each plasmid DNA mixture was prepared at the indicated concentrations in 250 µl of OPTI-MEM (Opti-MEM® Reduced-Serum Medium). The total amount of DNA in each transfection mixture was normalized by the addition of pcDNA3.1 vector (Invitrogen). To estimate the transfection efficiency, we used a cytomegalovirus promoter-driven enhanced green fluorescent protein expression vector pEGFP-C1 (Clontech, Mountain, CA). Each DNA mixture was incubated with 8 µl of Plus™ reagent at room temperature for 15 min and then with 2 µl of lipofectamine in an additional 250 µl of OPTI-MEM (Opti-MEM® Reduced-Serum Medium) for another 15 min. The transfection mixture was then applied to the cells. After 5 h incubation, the transfection medium was replaced with fresh growth medium containing serum, and cells were incubated overnight to allow for gene expression. Transfection efficiency was determined as the percentage of enhanced green fluorescent protein-positive cells, as assessed via fluorescent microscopy.

Small interfering RNAs

The small interfering RNA (siRNA) duplexes used in this study were purchased from Invitrogen and have the following sequences: Aurora A, AUGCCUGUCUUACUGUCATT; Aurora B, AGCAGUGGGACACCCGACAUCUUAA; Survivin, UUUAAAGCUGGGAGCCAGAUGACGC. BLOCK-iT Fluorescent Oligo (Invitrogen) or Negative Universal Control™ (Invitrogen) was used as the control. Cells were transfected with siRNA oligonucleotides using lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. To estimate the transfection efficiencies in the various siRNA experiments using siRNAs, we transfected cells with a fluorescently labeled control oligonucleotide (BLOCK-iT™ Fluorescent Oligo; Invitrogen) and assessed the percentage of green fluorescent cells via fluorescence microscopy. In all of our transfection experiments using siRNAs, the estimated transfection efficiencies (i.e. the percentages of green fluorescent cells) were >95%. To confirm successful siRNA-mediated knockdown, we performed western blotting of the proteins of interest.

Flow cytometry for the analysis of DNA contents

Trypsinized and floating cells were pooled, washed with phosphate-buffered saline (PBS) and fixed in 70% (vol/vol) ethanol. DNA contents were assessed by staining cells with propidium iodide after treatment with 50 µg/ml RNase A and monitoring by FACSscan (Becton Dickinson, Mountain View, CA). DNA content was assessed with a ModFit LT program (Verity Software House, Topsham, MA).

Immunocytochemistry

After treatments or siRNA transfection, cells were fixed with 4% paraformaldehyde for 20 min at room temperature, followed by permeabilization in 0.25% Triton X-100 for 5 min and blocking in 5% normal horse serum in PBS for 30 min. Fixed cells were incubated overnight at 4°C with primary antibody [anti- α -tubulin (1:200, mouse, Abcam), anti-lamin B (1:100, goat, Santa Cruz Biotechnology), anti-cleaved caspase-3 (1:200, rabbit, Cell Signaling), anti-HA (1:200, mouse, Covance) and anti-survivin (1:100, rabbit, Abcam)] diluted in PBS and then washed three times in PBS and incubated for 1 h at room temperature with anti-rabbit or anti-mouse Alexa Fluor 488 (1:200, Molecular Probes) or anti-rabbit or anti-mouse Alexa 594 was used as a secondary antibody (Molecular Probes). Next, cells were washed, stained with 1 µg/ml 4',6-diamidino-2-phenylindole for 10 min and washed with PBS. Slides were mounted with Pro-Long Gold antifade mounting reagent (Molecular probes) and cell staining was visualized with a fluorescence microscope (Axiovert 200M, Carl Zeiss).

RT-PCR analysis

Total RNA was extracted from Hep3B cells using the TRIzol reagent (Invitrogen). RT-PCR was done, following the manufacturer's protocol (TaKaRa Shuzo Co., Otsu, Shiga, Japan). The cDNAs were amplified by polymerase chain reaction (30 s at 94°C, 30 s at 60°C and 1 min at 74°C) with *Taq* DNA polymerase. Conditions for final analysis were chosen when amplification of messenger RNA (mRNA) was in the middle of the exponential amplification phase. Human survivin mRNA was amplified using the sense primer 5'-CAGATTTGAATCGCGGGACCC-3' and the antisense primer 5'-CCAGAGTCTGGCTCGTTCAG-3' (corresponding to a 206 bp region of survivin). For β -actin, the sense primer 5'-CAGGTCATCACCATTGGCAATGAGC-3' and the antisense primer 5'-GATGTCCACGTCACTTCATGA-3' (corresponding to a 132 bp region of β -actin) were used. The polymerase chain reaction cycling conditions (30 cycles) chosen were as follows: (i) 30 s at 94°C; (ii) 1 min at 60°C for survivin and 30 s at 60°C for β -actin and (c) 1 min 30 s at 72°C, with a subsequent 10 min extension at 72°C. Reaction products were analyzed on 2% agarose gels. The bands were visualized by ethidium bromide.

Statistical analysis and determination of synergy

All data are presented as mean \pm SE from at least three separate experiments. The statistical analyses were performed using SPSS v12.0.1 software (SPSS, Chicago, IL). We compared mean values using *T*-tests and analysis of variance with Bonferroni correction. All *P*-values <0.05 were considered to represent significant differences. The possible synergistic effect of ZM447439 and TRAIL was evaluated using the isobologram method. In brief, the cells were treated with different concentrations of ZM447439 and TRAIL alone or in combination. After 24 h, relative survival was assessed and the concentration-effect curves were used to determine the IC₅₀ (the half-maximal inhibitory concentration) values for each drug alone and in combination with a fixed concentration of the second agent.

Results

Aurora B contributes more than Aurora A to TRAIL resistance in cancer cells

Although TRAIL induces apoptosis and is considered a promising anti-cancer agent (1), many cancer cells are resistant to the apoptosis-inducing effect of TRAIL (2). Here, an examination of the effect of TRAIL on the viability of various cell lines showed that a number of hepatoma (HepG2, Hep3B and Huh-7), glioma (U251MG and SNU-201), breast cancer (Hs578T) and colon cancer (SW-837, RKO and HT-29) cell lines were very resistant to different doses of TRAIL (Figure 1A). In contrast, U343 and U251N glioma cells and HCT116 colon cancer cells were very sensitive to TRAIL, even at a dose as low as 25 ng/ml, whereas MDA-MB-435S breast cancer cells were partially sensitive to TRAIL. When we tested whether these differences in TRAIL resistance/sensitivity were associated with the expression levels of Aurora A and/or Aurora B, we found that the protein levels of Aurora A and Aurora B were lower in the TRAIL-sensitive cancer cell lines (U343, U251N and HCT116) than in the TRAIL-resistant cancer cell lines (Figure 1B).

To determine whether there was a functional connection between TRAIL sensitivity and the expression levels of Aurora A and/or Aurora B, we tested whether exogenously expressed Aurora A and/or Aurora B could confer TRAIL resistance to TRAIL-sensitive cancer cell lines. TRAIL-sensitive U343 and U251N glioma cells were transfected with expression vectors encoding Aurora A and/or Aurora B (or a control vector) and then treated with 50 ng/ml TRAIL. We found that TRAIL-induced cell death was more strongly attenuated by overexpression of Aurora B versus Aurora A (Figure 1C). However, the co-overexpression of Aurora A and Aurora B did not greatly enhance the abrogation of TRAIL-induced cell death over that caused by Aurora B alone. We then addressed the role of Aurora A and Aurora B in TRAIL sensitivity by examining whether TRAIL sensitivity could be restored by knocking down Aurora A or Aurora B expression in TRAIL-resistant cancer cells. We transfected TRAIL-resistant Hep3B cells with Aurora A and/or Aurora B siRNA (or a control oligonucleotide) and then treated the cells with TRAIL. As shown in Figure 1D, siRNA-mediated Aurora B suppression recovered TRAIL sensitivity in Hep3B cells more effectively than siRNA-mediated knockdown of Aurora A. Moreover, the Aurora B siRNA-mediated sensitization of cells to TRAIL-mediated apoptosis was only slightly enhanced by cotransfection with Aurora A siRNA.

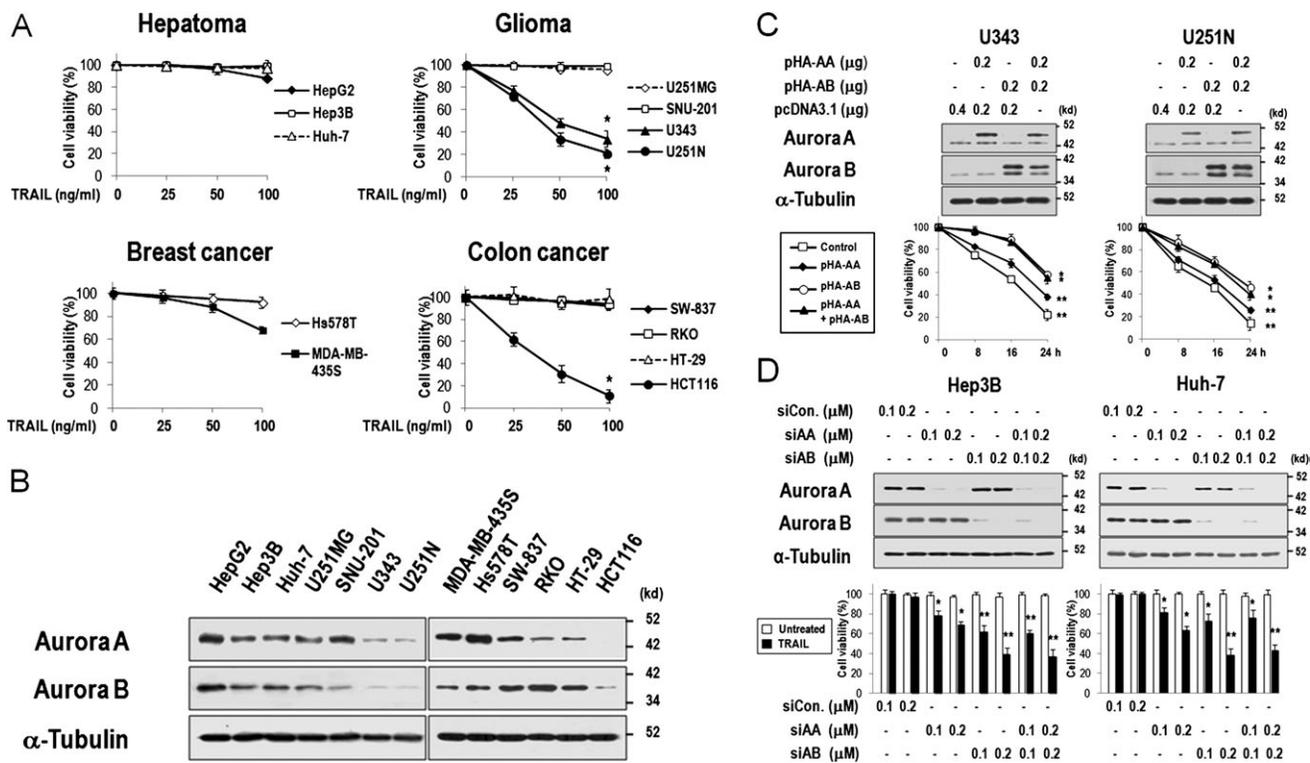


Fig. 1. Aurora B contributes more highly than Aurora A to TRAIL resistance in cancer cells. TRAIL sensitivity in various cancer cells may be associated with the expressions of Aurora A and/or Aurora B. (A) Cells were treated with the indicated concentrations of TRAIL for 24 h, and cellular viabilities were measured using calcein-AM and ethidium homodimer-1. Symbols: points, average of three independent experiments; bars, SE; * $P < 0.001$ versus untreated cells. (B) Cell extracts were prepared for western blotting of Aurora A and Aurora B. To confirm equal loading of protein samples, western blotting of α -tubulin was performed. (C) TRAIL-sensitive U343 and U251N cells were transiently transfected with the indicated concentrations of pHA-AA, pHA-AB or pcDNA3.1 using Lipofectamine PLUS (Invitrogen) according to the manufacturer's recommendations. The transfection efficiencies of the U343 and U251N cells, which were assessed by transfection of an enhanced green fluorescent protein-expressing vector, were 37 and 34%, respectively. Overexpression of Aurora A or Aurora B was confirmed by western blotting using anti-Aurora A and anti-Aurora B antibodies, respectively. The transfected cells were treated with 50 ng/ml TRAIL for the indicated time points, and cellular viability was measured using calcein-AM and ethidium homodimer-1. Points, average of three independent experiments; bars, SE; * $P < 0.01$; ** $P < 0.001$ versus untreated cells. (D) Hep3B or Huh-7 cells were transfected with the control oligonucleotide (siCon.; Negative Universal ControlTM) or siRNAs targeting Aurora A and/or Aurora B. Transfection efficiency, which was judged by employing BLOCK-iTTM Fluorescent Oligo, was $>95\%$. siRNA-mediated suppression of Aurora A or Aurora B was confirmed by western blotting. The transfected cells were further treated with 200 ng/ml TRAIL for 24 h, and cellular viability was assessed using calcein-AM and ethidium homodimer-1. Columns, average of three independent experiments; bars, SE; * $P < 0.01$; ** $P < 0.001$ versus cells transfected with 0.1 μ M siCon. and further treated with TRAIL.

Similar results were obtained in Huh-7 cells transfected with Aurora A siRNA and/or Aurora B siRNA (Figure 1D). Taken together, these results indicate that Aurora B contributes more than Aurora A to TRAIL resistance in cancer cells.

Inhibition of Aurora B kinase activity effectively sensitizes various TRAIL-resistant cancer cells to TRAIL-mediated apoptosis

Next, we investigated whether the enhancement of TRAIL sensitivity by Aurora B knockdown was associated with changes in caspase activity. Consistent with our previous report (23), in Hep3B cells treated with TRAIL alone, the 32 kDa pro-caspase-3 was partially cleaved to a 20 kDa intermediate form, but further cleavage into the p17 active subunit was not detected. Under the same conditions, there was no apparent processing of pro-caspase-8 and pro-caspase-9 or PARP (a representative substrate of caspase-3). In Aurora B knockdown cells, however, we observed TRAIL-mediated proteolytic processing of pro-caspase-3 into the p17 active subunit (Figure 2A) as well as effective processing of PARP. We then tested whether ZM447439 (ZM, a specific inhibitor of Aurora B) (24) mimicked the effect of Aurora B siRNA on TRAIL-mediated processing of caspase-3. Inhibition of Aurora B kinase activity by ZM447439 was confirmed in HepG2 cells by western blotting using a phospho-histone H3 antibody to detect phosphorylated serine 10 in histone H3, a physiological target of Aurora B (25) (Figure 2B). Further examination revealed that although treatment with 10 μ M ZM447439 alone did not induce proteolytic processing of cas-

pase-3 in these cells, combined treatment with ZM447439 and TRAIL induced the complete processing of caspase-3 into the p17 active subunit. We next examined whether TRAIL resistance in various cancer cells might also be overcome by cotreatment with a specific inhibitor of Aurora B kinase activity. Treatment of various TRAIL-resistant cancer cells with ZM447439 alone had no appreciable effect on cell viability at concentrations of ZM447439 up to 10 μ M (Figure 2C). In contrast, combined treatment of these cells with a fixed concentration of TRAIL and varied concentrations of ZM447439 or, conversely, with a fixed concentration of ZM447439 and varied concentrations of TRAIL significantly reduced cell viability. When combined with ZM447439 and TRAIL effectively induced cell death in several colon cancer cell lines (e.g. RKO and SW-837) at lower concentrations than those were required to induce cell death in many hepatoma, glioma and breast cancer cells. Furthermore, an isobologram analysis (26) demonstrated that there were synergistic interactions between ZM447439 and TRAIL in Hep3B, HepG2 and U251MG cells (Figure 2D). Taken together, these results suggest that this combined regimen may offer an attractive strategy for effectively treating malignant cancer cells.

Survivin may be a target of Aurora B in mediating the TRAIL resistance of cancer cells

We then investigated some potential underlying mechanisms through which knockdown or inhibition of Aurora B could sensitize cancer cells to TRAIL-mediated apoptosis. Li *et al.* (27) recently showed that

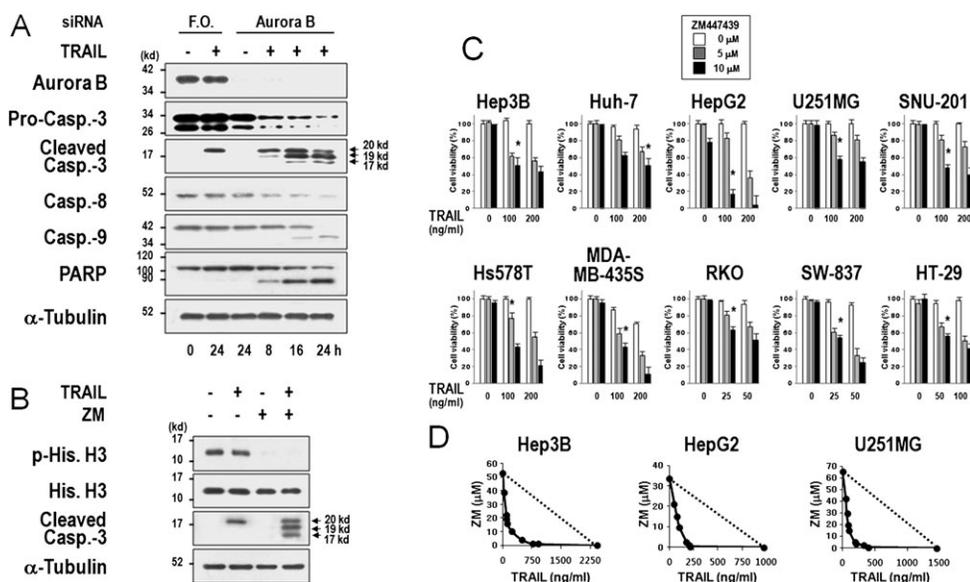


Fig. 2. Both the suppression of Aurora B expression and the inhibition of Aurora B kinase by ZM447439 sensitize various cancer cells to TRAIL-mediated apoptosis. (A) Hep3B cells were transfected with the control fluorescent oligonucleotide (FO) or Aurora B siRNA and further treated with or without 200 ng/ml TRAIL for the indicated time points. Transfection efficiency, which was judged by employing the control fluorescent oligonucleotide, was >95%. Western blotting was used to detect changes in the proteolytic processing of caspases and PARP. Western blotting of α -tubulin was used to show equal loading of protein samples. (B) HepG2 cells were treated with 100 ng/ml TRAIL alone, 10 μ M ZM447439 (ZM) alone or both together. After 24 h, Aurora kinase activity was assessed by western blot analysis of the phosphorylation levels of histone H3 at serine 10. Western blotting was also used to detect cleaved caspase-3 and α -tubulin. (C) Cells were treated with ZM447439 for 30 min and further treated with or without the indicated concentrations of TRAIL for 24 h. Cellular viabilities were measured using calcein-AM and ethidium homodimer-1. Columns, average of three independent experiments; bars, SE; * P < 0.001 compared with untreated cells. (D) Hep3B, HepG2 or U251MG cells were exposed to ZM447439 alone, TRAIL alone or cotreated with various dilutions of the two agents for 24 h. Isoboles were obtained by plotting the combined concentrations of each drug required to produce 50% cell death. The straight line connecting the IC₅₀ values obtained for two agents when applied alone corresponds to an additivity of their independent effects. Values below this line indicate synergy, whereas values above this line indicate antagonism.

inhibition of Aurora B kinase sensitized a subset of human glioma cells to TRAIL by inducing the TRAIL receptor, DR5. However, we found that the protein levels of DR5 were not altered by Aurora B knockdown in Hep3B cells (Figure 3A). Other anti-apoptotic proteins, including c-IAP2, Bcl-2 and Bcl-xL, were also unaffected by siRNA-mediated suppression of Aurora B. In contrast, the protein levels of survivin were remarkably reduced by siRNA-mediated suppression of Aurora B, whereas those of XIAP were slightly reduced. We further tested whether the expression of these anti-apoptotic proteins is similarly regulated in Huh-7 cells transfected with Aurora B siRNA. We found that Bcl-2 was barely detected in Huh-7 cells, whereas the protein levels of DR5, XIAP, c-IAP2 and Bcl-xL were not altered by Aurora B knockdown (Figure 3B). Interestingly, the protein levels of survivin were also markedly reduced in these cells following Aurora B knockdown (Figure 3B), indicating that survivin may be a common target in cancer cells subjected to Aurora B knockdown. We next examined whether ZM447439 sensitized TRAIL-resistant cancer cells to TRAIL-induced apoptosis via the same mechanism triggered by Aurora B knockdown. We found that survivin protein levels were markedly downregulated by ZM447439 treatment in all tested cancer cell lines (Figure 3C). To confirm the functional significance of this downregulation of survivin in the sensitization of TRAIL-mediated apoptosis, we examined whether the suppression of survivin expression could enhance TRAIL-mediated apoptosis. We found that siRNA-mediated survivin knockdown significantly reduced the viability of TRAIL-treated Hep3B cells, whereas Hep3B cells transfected with the control siRNA were not affected by TRAIL treatment (Figure 3D). In addition, the TRAIL-induced partial processing of caspase-3 seen in Hep3B cells was completed in cells cotreated with TRAIL and the survivin siRNA. These results suggest that survivin may be a target of Aurora B in mediating the TRAIL resistance of cancer cells.

Mitotic catastrophe induced by inhibition of Aurora B may contribute to the increase in TRAIL sensitivity

Both Aurora B and survivin are important regulators of the mitotic spindle checkpoint system, which is a cellular surveillance mechanism

that prevents the progression of mitosis until all chromosomes are properly aligned during metaphase (14). Here, we sought to examine whether the Aurora B inhibition-induced sensitization of cells to TRAIL-mediated apoptosis was associated with changes in cell cycle distribution. Hep3B cells were treated with ZM447439 and/or TRAIL and subjected to fluorescence activated cell sorting analysis (Figure 4A), and the percentages of 2N, 4N, polyploid (DNA content > 4N) and hypoploid cells were quantified (Figure 4B). Treatment with 100 ng/ml TRAIL alone did not alter the cell cycle distribution. In cells treated with 10 μ M ZM447439 for 24 h, the percentage of polyploid cells was markedly increased, whereas the percentage of 2N cells was reduced. Following the prolonged exposure of Hep3B cells to ZM447439 for 48 h, the percentage of polyploid cells was further increased, and the hypoploid apoptotic cell population (6.8%) was slightly increased. We also observed increased polyploid cell populations in Hep3B cells transfected with Aurora B or survivin siRNA (Supplementary Figure 2A–C is available at *Carcinogenesis* Online), although these increases were less dramatic than those seen in cells treated with ZM447439 alone (Figure 4A and B). Combined treatment with ZM447439 and TRAIL for 24 h increased the polyploid cell population to an extent similar to that seen in cells treated with ZM447439 for 24 h but further increased the hypoploid cell population (13.5%) (Figure 4B). To examine the nuclear morphologies of the polyploid and hypoploid cells that were increased following treatment with ZM447439 and/or TRAIL, we performed 4',6-diamidino-2-phenylindole staining and immunocytochemical analysis of α -tubulin. We found that treatment of Hep3B cells with 10 μ M ZM447439 for 24 h induced multinucleation and micronucleation with decondensed chromatin (which are all characteristics of mitotic catastrophe) in most of the treated cells (Figure 4C). Although treatment with 100 ng/ml TRAIL alone did not alter the nuclear morphologies of the treated cells, multinucleation and micronucleation were observed in Hep3B cells cotreated with ZM447439 and TRAIL. Interestingly, marked increases of apoptotic bodies with condensed chromatin were frequently observed on the surfaces of the multinucleated and micronucleated cells (Figure 4C), suggesting that the apoptotic

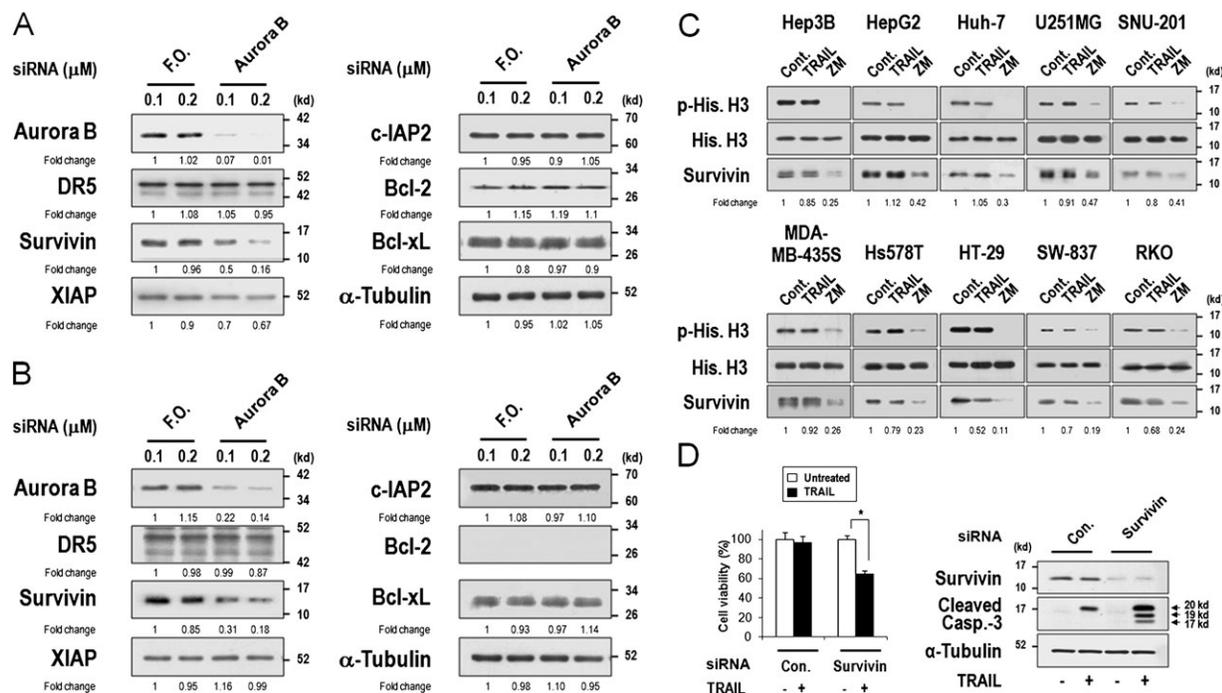


Fig. 3. Both the suppression of Aurora B expression and the inhibition of Aurora B activity by ZM447439 downregulate survivin. Hep3B (A) and Huh-7 (B) cells were transfected with the indicated concentrations of the control fluorescent oligonucleotide (FO) or Aurora B siRNA and cell extracts were prepared for western blotting of the indicated proteins. Transfection efficiency, which was judged by employing the control fluorescent oligonucleotide, was >95%. Numbers indicate the fold change of proteins compared with the control. Similar results were obtained from three independent experiments in each case. (C) Various cancer cells were treated with 100 ng/ml TRAIL or 10 μM ZM447439 for 24 h, and cell extracts were prepared for western blotting of the indicated proteins. (D) Hep3B cells were transfected with the control oligonucleotide (Con.) or survivin siRNA and further treated with or without 200 ng/ml TRAIL for 24 h. Transfection efficiency, which was judged by employing the control fluorescent oligonucleotide, was >95%. Cell viability was assessed using calcein-AM and ethidium homodimer-1 (left). Columns, average of three independent experiments; bars, SE; * $P < 0.01$ versus untreated cells. Cell extracts were prepared for western blotting of survivin, cleaved caspase-3 and α -tubulin (right).

bodies might be derived from cells undergoing mitotic catastrophe following treatment with ZM447439 and TRAIL. Furthermore, immunocytochemistry using antibodies against lamin B (to detect the nuclear membrane) and cleaved caspase-3 showed that the apoptotic bodies on the multinucleated and micronucleated cells, as well as some of the multinucleated cells with condensed chromatin, were strongly positive for cleaved caspase-3 (Figure 4D). Thus, our results demonstrate that inhibition of Aurora B in cancer cells induces mitotic catastrophe, which is not enough to induce cell death. In contrast, combined treatment with the Aurora B inhibitor and TRAIL induces mitotic catastrophe leading to apoptosis. These findings suggest that mitotic catastrophe induced by Aurora B inhibition may contribute to enhancing TRAIL sensitivity in TRAIL-resistant cancer cells.

Phosphorylation of survivin by Aurora B contributes to its upregulation and anti-apoptotic effect on TRAIL-induced apoptosis

Since both siRNA-mediated Aurora B knockdown and ZM447439-mediated inhibition of Aurora B kinase activity effectively reduced the protein levels of survivin (Figure 3A–C), we next investigated whether Aurora B overexpression could upregulate the expression of survivin. Western blotting analysis showed that exogenous expression of Aurora B in Hep3B cells dose dependently increased the endogenous protein levels of survivin (Figure 5A). We further compared the expression patterns of endogenous survivin in the presence or absence of exogenously expressed Aurora B. Hep3B cells were transfected with pcDNA3.1 (control vector) or a vector encoding HA-tagged Aurora B, and immunocytochemistry was performed using anti-HA and anti-survivin antibodies. HA-Aurora B was expressed in both the nucleus and the cytosol, with higher expression levels seen in the nuclei (Figure 5B). Survivin was weakly but mainly expressed in the nuclei of Hep3B cells (transfected with pcDNA3.1). In contrast, survivin was highly expressed in both the nuclei and the cytosols of cells expressing HA-Aurora B. To explore the mechanism

underlying the Aurora B-mediated upregulation of survivin, we tested whether survivin expression was controlled at the transcriptional level. RT-PCR analysis demonstrated that neither Aurora B overexpression nor ZM447439 treatment altered the mRNA levels of survivin (Figure 5C), indicating that Aurora B-mediated survivin upregulation may be controlled at the post-transcriptional level. Previously, Aurora B was shown to phosphorylate survivin at threonine 117 (28), thereby negatively regulating the function of survivin during mitosis (29). We therefore tested whether the Aurora B-mediated upregulation of survivin was dependent on the Aurora B-mediated phosphorylation of survivin threonine 117. We transfected Hep3B cells with the plasmids encoding WT survivin or non-phosphorylatable survivin mutant (T117A), with or without cotransfection with different doses of a plasmid encoding HA-tagged Aurora B. We found that the protein levels of WT survivin were dose dependently increased by coexpression of Aurora B, but the protein levels of the T117A mutant survivin were unaltered under these conditions (Figure 6A). Immunocytochemical analysis showed that the exogenously expressed survivin was expressed in both the nucleus and the cytosol, with slightly higher levels seen in the nucleus (Figure 6B). Coexpression of Aurora B and WT survivin remarkably increased the expression of survivin, particularly near areas of high Aurora B expression. In contrast, the distribution and level of T117A mutant survivin expression appeared unchanged by the coexpression of Aurora B. Collectively, these results suggest that phosphorylation of survivin by Aurora B may lead to the Aurora B-mediated enhancement of survivin expression. Next, we investigated whether TRAIL sensitivity is affected by survivin depending on its phosphorylation status at threonine 117, using MDA-MB-435S cells with moderate TRAIL sensitivity (Figure 1A). After transfection of MDA-MB-435S cells with the plasmids encoding WT survivin or non-phosphorylatable survivin mutant (T117A), we compared the effect of TRAIL on the viabilities of these transfected cells. We found that WT survivin dose dependently attenuated

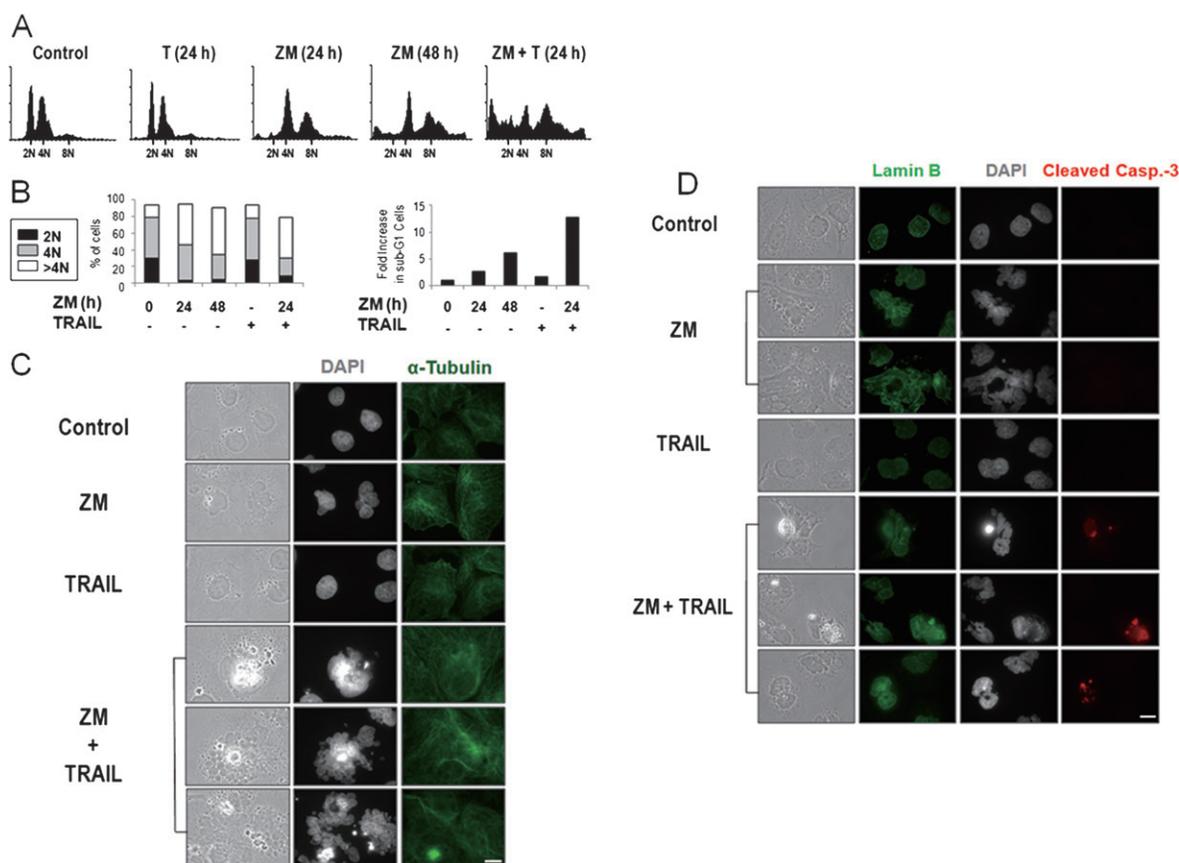


Fig. 4. Mitotic catastrophe induced by Aurora B inhibition may sensitize Hep3B cells to TRAIL-mediated apoptosis. (A) Hep3B cells were treated for the indicated times with 10 μ M ZM447439 and/or 100 ng/ml TRAIL, stained with propidium iodide and subjected to flow cytometric analysis of DNA contents. (B) The percentages of 2N, 4N, polyploid (DNA content > 4N) and hypoploid cells were plotted for each treatment group based on the flow cytometric data from (A). (C and D) Hep3B cells were treated with 10 μ M ZM447439 and/or 100 ng/ml TRAIL for 24 h and then subjected to immunocytochemical analysis of α -tubulin (green) and nuclei (gray) (C) or lamin B (green), cleaved caspase-3 (red) and nuclei (gray); bar, 2 μ m (D).

TRAIL-induced cell death as well as the cleavage of caspase-3 and PARP in MDA-MB-435S cells, whereas T117A mutant survivin did not (Figure 6C and D). These results suggest that phosphorylation of survivin at threonine 117 may contribute to not only its upregulation but also its anti-apoptotic activity against TRAIL-induced apoptosis.

Collectively, our results suggest that Aurora B, which is highly expressed in many cancer cells, triggers the phosphorylation and enhanced protein expression of survivin, conferring cancer cells resistance to TRAIL-mediated apoptosis. Thus, the inhibition of Aurora B kinase activity can sensitize TRAIL-resistant cancer cells to TRAIL-mediated apoptosis by downregulating survivin.

Discussion

TRAIL is considered a promising candidate for medical applications because it triggers apoptosis preferentially in cancer cells, whereas showing little or no toxicity toward normal cells (1). However, its therapeutic efficacy has been limited by the TRAIL resistance of many cancer cells, including human hepatoma and glioma cells (2,13,30). TRAIL resistance in cancer cells appears to occur through various molecular mechanisms, including differential expression of death receptors, high Akt and nuclear factor-kappaB activity levels, overexpression of c-FLIP, mutations in Bax and Bak and defects in the release of mitochondrial proteins (31). Moreover, high-level expression of the inhibitor of apoptosis proteins, such as survivin and XIAP, which block apoptosis at the effector phase (a point at which multiple signaling pathways converge), may also contribute to TRAIL resistance (31). In this study, we clearly demonstrate that Aurora B is an important contributor to TRAIL resistance in cancer cells.

The Aurora kinases are highly expressed in various types of tumors (e.g. colon, breast, ovarian, gastric and pancreatic tumors) compared with matched normal tissues, and Aurora expression levels often correlate with tumor characteristics and/or clinical outcomes (8–13,32). These kinases have received considerable attention as potential targets of new cancer therapeutics due to their involvements in mitotic control, genomic instability and tumorigenesis (7,14). In addition, a recent study found that Aurora A overexpression notably enhanced the resistance of various cancer cells to chemotherapy-induced apoptosis (15–17), suggesting that the anti-apoptotic activity of Aurora A may contribute to its oncogenic properties. Unlike the case of Aurora A, however, the apoptosis-modulating activity of Aurora B has not been extensively investigated. In this study, we clearly show that Aurora B is critically involved in conferring TRAIL resistance to various cancer cells. This conclusion is based on the following evidence: (i) Aurora B was found to be highly expressed in various TRAIL-resistant cancer cells but not in TRAIL-sensitive cancer cells; (ii) Overexpression of Aurora B effectively attenuated TRAIL-induced apoptosis in several TRAIL-sensitive cancer cell lines, including U343 and U251N cells; (iii) TRAIL-resistant Hep3B and Huh-7 cells were significantly sensitized to TRAIL-induced apoptosis by siRNA-mediated suppression of Aurora B expression. Notably, the anti-apoptotic activity of Aurora B against TRAIL-induced cell death was higher than that of Aurora A.

ZM447439, which was the first developed Aurora kinase inhibitor (24), inhibits the phosphorylation of serine 10 in histone H3, which is a physiological target of Aurora B (25). A recent study found that ZM447439 selectively inhibited Aurora B over Aurora A (33). Recently, another Aurora B kinase inhibitor, AZD1152, was shown to sensitize a subset of human glioma cells to TRAIL concomitant with the induction of DR5 (27). Here, we found that subtoxic doses of

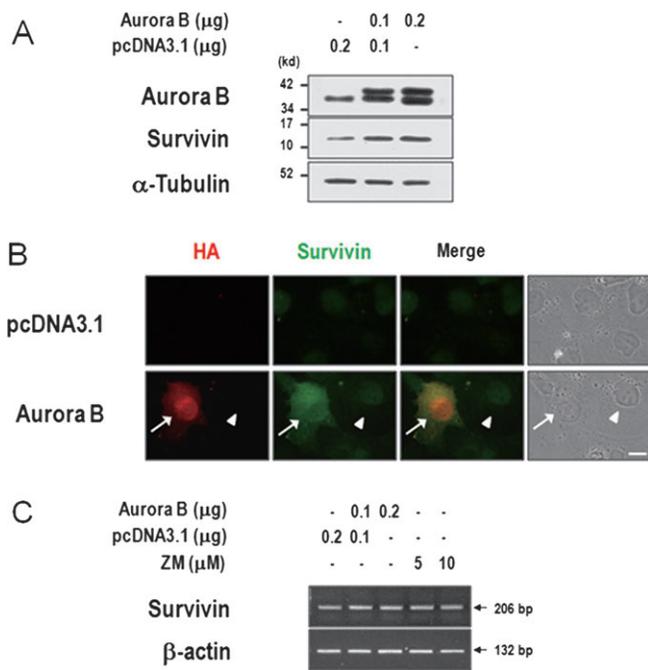


Fig. 5. Aurora B upregulates survivin protein levels at the post-transcriptional level. (A) Hep3B cells were transfected with the indicated concentrations of pcDNA3.1 or the expression vector encoding HA-tagged Aurora B and then subjected to western blotting of Aurora B, survivin and α -tubulin. (B) Hep3B cells were transfected with 0.2 μ g pcDNA3.1 or the HA-tagged Aurora B-encoding plasmid, fixed and subjected to immunocytochemistry using anti-HA or anti-survivin antibodies. Arrow and arrowhead indicate the cells transfected with HA-Aurora B and untransfected cells, respectively; bar, 2 μ m. (C) Total RNAs were isolated from Hep3B cells transfected with the expression vector encoding Aurora B or pcDNA3.1 as well as Hep3B cells treated with ZM447439 for the indicated concentrations. RT-PCR analysis of survivin and β -actin was performed.

ZM447439 effectively sensitized a variety of TRAIL-resistant cancer cells to TRAIL-induced apoptosis by inhibiting Aurora B kinase. However, upregulation of DR5 was not a consistent phenomenon in our system (Supplementary Figure 1 is available at *Carcinogenesis* Online). Increase in DR5 protein levels by ZM447439 was marked in several types of TRAIL-resistant cancer cells (e.g. Hs578T, RKO, U251MG, SNU-201 and HT-29 cells) but not in other cancer cells (e.g. MDA-MB-435S, SNU-449 and SW-837 cells). At present, the molecular basis for the variable effect of ZM447439 on DR5 expression in different cancer cell lines is not clear.

Unlike the case of DR5, however, we found that survivin protein levels were considerably downregulated in Hep3B cells transfected with Aurora B siRNA, as well as in all the tested TRAIL-resistant cancer cell lines following ZM447439 treatment, demonstrating the functional significance of survivin downregulation. Survivin, an inhibitor of apoptosis, acts as a chromosomal passenger protein for proper chromosomal segregation with Aurora B (34,35). Significant survivin overexpression in various types of cancer has been correlated with reduced tumor cell apoptosis, increased resistance to cancer therapy and abbreviated patient survival (36–39). Many research groups have extensively explored the functional links between Aurora B and survivin during mitosis. For example, survivin dynamics have been shown to increase at the centromeres during the G₂/M phase transition under regulation by Aurora B kinase activity (40). In addition, Aurora B kinase activity was found to be stimulated by the binding and phosphorylation of survivin (34,41), suggesting the existence of cross-talk between these proteins during the regulation of mitosis. Both Aurora B and survivin are important regulators of the mitotic spindle checkpoint system (15). Inhibition of either Aurora B or survivin reportedly blocks the completion of cell division; cells

continue to enter and exit mitosis with regular kinetics but do not undergo appropriate cytokinesis, leading to the accumulation of polyploid cells (24,42). In addition, the inhibition of either Aurora B or survivin has been shown to induce mitotic catastrophe in various cancer cells (43,44). Consistent with these previous results, we found that treatment of Hep3B cells with ZM447439 induced polyploidy and mitotic catastrophe (Figure 4). We further found that knockdown of either Aurora B or survivin in Hep3B cells also increased polyploidy (Supplementary Figure 2A–C is available at *Carcinogenesis* Online) and mitotic catastrophe (Supplementary Figure 2D is available at *Carcinogenesis* Online), although to a lesser extent than that seen following ZM447439 treatment (Figure 4). It was recently proposed that mitotic catastrophe might not even constitute a bona fide cell death executioner mechanism, but an oncosuppressive pathway that precedes and is distinct from, yet operates through, cell death or senescence (45). The Hep3B cells used in the present study did not show significant cell death following treatment with TRAIL alone as well as after induction of mitotic catastrophe by ZM447439 treatment, Aurora B knockdown or survivin knockdown alone. In contrast, Hep3B cells treated with ZM447439 or transfected with Aurora B or survivin siRNA, and then further treated with TRAIL, all showed increases in sub-G₁ apoptotic cell populations (Figure 4A and B and Supplementary Figure 2A–C is available at *Carcinogenesis* Online). Notably, many apoptotic bodies with highly condensed chromatin were detected on the multinucleated and micronucleated cells observed among not only Hep3B cells treated with ZM447439 plus TRAIL (Figure 4C) but also Hep3B cells treated with Aurora B siRNA plus TRAIL (Supplementary Figure 3A and B is available at *Carcinogenesis* Online). Furthermore, strong caspase-3 activity was detected in these apoptotic bodies (Figure 4D and Supplementary Figure 3B, available at *Carcinogenesis* Online). In cells treated with ZM447439 and TRAIL for 24 h, the loss of viability (~50%, Figure 2C) was much higher than the increase in the sub-G₁ apoptotic cell population (13.5%, Figure 4B). This discrepancy could possibly indicate that our fluorescence activated cell sorting analysis may have sorted many of the adhered caspase-3-positive apoptotic bodies as polyploid cells but not sub-G₁ apoptotic cells. Taken together, our results suggest that although the mitotic catastrophe induced by Aurora B inhibition is not sufficient to trigger cell death, it can sensitize TRAIL-resistant cancer cells to TRAIL-mediated apoptosis. In this process, the downregulation of survivin by Aurora B inhibition is presumed to contribute to both mitotic catastrophe and caspase-3 activation.

Here, we provide direct evidence that Aurora B contributes to TRAIL resistance in cancer cells via phosphorylation of survivin. The protein levels of survivin were dose dependently increased by Aurora B overexpression, but the mRNA levels of survivin were not altered by either Aurora B overexpression or ZM447439 treatment (Figure 5). Previously, phosphorylation of survivin at residue threonine 117 by Aurora B kinase was shown to regulate the formation of the chromosomal passenger complex consisting of Aurora B, inner centromere protein, survivin and borealin, which contribute to the proper mitotic process (46). Thus, we tested whether the Aurora B-mediated upregulation of survivin was also associated with survivin phosphorylation at residue threonine 117. Interestingly, forced overexpression of Aurora B increased the protein levels of WT survivin, but not those of the non-phosphorylatable mutant survivin (T117A), suggesting that the phosphorylation of survivin by Aurora B is responsible for the noted increase in survivin protein expression in Aurora B-overexpressing cells. As shown in Figure 5B, our immunocytochemical analysis revealed that the expression of endogenous survivin was mainly nuclear in the absence of forced overexpression of Aurora B, whereas highly enhanced expression of endogenous survivin was seen in both the nuclei and the cytosols of Aurora B-overexpressing cells. When we cotransfected Hep3B cells with plasmids encoding Aurora B and WT survivin, the expression levels of survivin also increased in both the nucleus and the cytosol. Replacement of survivin threonine 117 with alanine abrogated a phosphorylation site but did not dramatically alter the cellular localization of survivin (Figure 6B). Notably, the expression level of this survivin mutant was unaffected by Aurora

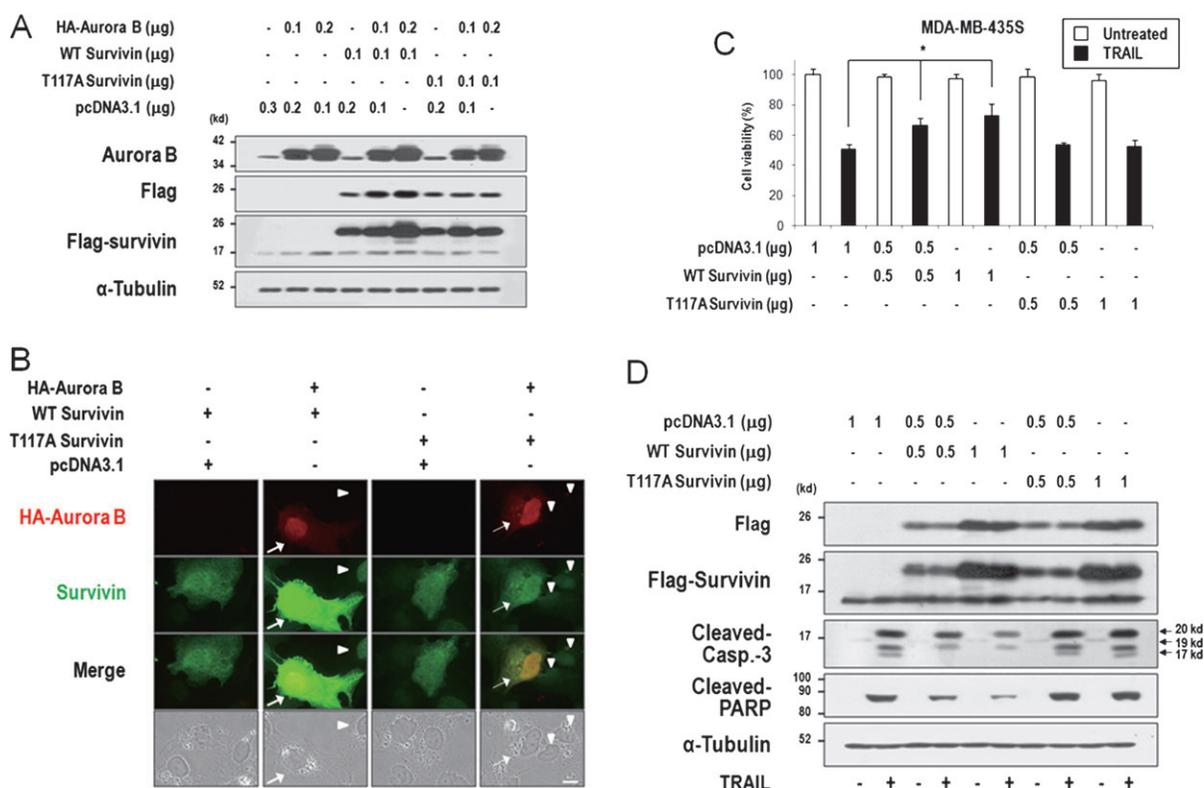


Fig. 6. Phosphorylation of survivin at threonine 117 by Aurora B is important for both its upregulation and its anti-apoptotic activity against TRAIL-induced apoptosis. (A) Hep3B cells were transfected with the plasmid encoding Flag-tagged WT survivin or Flag-tagged T117A mutant survivin (T117A) with or without the plasmid encoding HA-tagged Aurora B and pcDNA3.1. Cell extracts were subjected to western blotting of the indicated proteins. (B) Hep3B cells were transfected with 0.2 μg of the WT survivin plasmid or T117A mutant survivin plasmid together with pcDNA3.1 or the HA-Aurora B-encoding plasmid. After fixation, the cells were subjected to immunocytochemistry using anti-HA or anti-survivin antibodies. Arrow and arrowhead indicate HA-Aurora B-transfected and untransfected cells, respectively; bar, 2 μm. (C) MDA-MB-435S cells were transfected with pcDNA3.1, the plasmid encoding Flag-tagged WT survivin or Flag-tagged T117A mutant survivin (T117A) at the indicated amount. The transfection efficiency in MDA-MB-435S cells, which was assessed by transfection of an enhanced green fluorescent protein-expressing vector, was ~55%. These transfected cells were treated with 100 ng/ml TRAIL for 36 h. Cell viability was assessed using calcein-AM and ethidium homodimer-1. Columns, average of three independent experiments; bars, SE; * $P < 0.01$ versus cells transfected with 1 μg pcDNA3.1 and further treated with TRAIL. (D) Cell extracts were prepared for western blotting of survivin, cleaved caspase-3, cleaved PARP and α-tubulin.

B overexpression, indicating that the phosphorylation of survivin was required for the Aurora B-mediated upregulation of survivin. Survivin, which has a nuclear export signal in the linker region between BIR (baculovirus inhibitor of apoptosis repeat) domain and the COOH-terminal alpha helix, has been shown to shuttle between the cytoplasm and nucleus (47) and is reportedly found in both the cytoplasm and the nuclei of interphase cells from cancer patients (48,49). The cytoplasmic portion of survivin is thought to confer an anti-apoptotic function by directly or indirectly interfering with caspase function, whereas nuclear survivin is suspected to control cell division (50). We further attempted to investigate whether phosphorylation of survivin by Aurora B is associated with its anti-apoptotic activity against TRAIL-induced apoptosis. As shown in Figure 6C and D, WT survivin but not non-phosphorylatable mutant survivin (T117A) dose dependently inhibited TRAIL-induced apoptosis in MDA-MB-435S cells, reducing the cleavage of caspase-3 and PARP. These results suggest that Aurora B-mediated survivin phosphorylation may contribute to not only the increase in its cytoplasmic concentration but also the anti-apoptotic activity against TRAIL-induced apoptosis. Further studies should test whether Aurora B-mediated phosphorylation/upregulation of survivin increases its nuclear export and/or inhibits its degradation in the cytosol. In addition, it remains to be clarified whether phosphorylation of survivin by Aurora B directly affects its binding affinity to caspases.

In conclusion, we herein propose for the first time that TRAIL resistance in various cancer cells is closely associated with enhanced Aurora B activity and that survivin is a target of Aurora B in this process. Therefore, inhibition of Aurora B in these cancer cells can

restore sensitivity to TRAIL-mediated apoptosis via the downregulation of survivin. Future studies confirming the observed phenomena in *ex vivo* tumor cells could help us predict the efficacy of Aurora B inhibition/TRAIL cotreatments against cancers in the clinical setting.

Supplementary material

Supplementary Figures 1–3 can be found at <http://carcin.oxfordjournals.org/>

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