

## The Suppression of *Aurora-A*/*STK15*/*BTAK* Expression Enhances Chemosensitivity to Docetaxel in Human Esophageal Squamous Cell Carcinoma

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**Abstract Purpose:** We previously reported that the expression of Aurora-A was frequently up-regulated in human esophageal squamous cell carcinoma (ESCC) tissues as well as cell lines and the up-regulation contributed to a poor prognosis. In this study, we assessed the possibility of Aurora-A suppression as a therapeutic target for ESCC using ESCC cell lines.

**Experimental Design:** We established subclones using vector-based short hairpin RNA (shRNA). Then, we investigated the effect of Aurora-A suppression on proliferation and cell cycle changes *in vitro*. Next, chemosensitivity against docetaxel was investigated by tetrazolium salt-based proliferation assay (WST assay) and cell number determinations, and furthermore, the type of cell death induced by docetaxel was analyzed by flow cytometry. Finally, to examine the effect of Aurora-A shRNA on proliferation and chemosensitivity against docetaxel *in vivo*, a s.c. tumor formation assay in nude mice was done.

**Results:** We established two genetically different stable cell lines (510 A and 1440 A) in which levels of Aurora-A were reduced. Cell growth was inhibited by 38.7% in 510 A and by 24.3% in 1440 A *in vitro* compared with empty vector-transfected controls (510 m and 1440 m), and this growth inhibition was mediated through G<sub>2</sub>-M arrest as confirmed by flow cytometry. Next, in a WST assay, the IC<sub>50</sub> for Aurora-A shRNA-transfected cells was lower than that of empty vector-transfected cells (510 A,  $2.7 \times 10^{-7}$  mol/L; 510 m,  $4.8 \times 10^{-7}$  mol/L; 1440 A,  $2.6 \times 10^{-7}$  mol/L; 1440 m,  $4.9 \times 10^{-7}$  mol/L). In addition, 0.3 nmol/L docetaxel induced a notable level of apoptosis in Aurora-A shRNA-transfected cells compared with empty vector-transfected cells. In the assay of s.c. tumors in nude mice, tumor growth in 510 A was inhibited by 36.1% compared with that in 510 m, and in tumors treated with docetaxel, the suppression of Aurora-A resulted in 44.0% tumor growth suppression *in vivo*.

**Conclusions:** These results indicated that Aurora-A might play an important role in chemosensitivity to docetaxel, and the suppression of its expression might be a potential therapeutic target for ESCC.

Several proteins strictly regulate the process of cellular division. Defects in chromatid segregation cause genetic instability, a condition associated with tumorigenesis. During the proliferation of normal cells, the centrosome ensures the equal

segregation of chromosomes to the postmitotic daughter cells by organizing the bipolar mitotic spindle. In contrast, in cancer cells, multipolar mitotic spindles and various centrosomal anomalies, such as supernumerary centrosomes, centrosomes of abnormal size and shape, aberrantly phosphorylated centrosomic proteins, and prematurely split centrosomes, are frequently observed (1–7).

Aurora-A, a member of the Aurora/Ipl1p family of cell cycle-regulating serine/threonine kinases, is expressed at interphase mitotic centrosomes and the spindle poles in the nucleus where it regulates segregation of chromosomes and cytokinesis. Recent studies have shown that the ectopic expression of Aurora-A in mouse NIH/3T3 cells and Rat 1 fibroblasts causes centrosome amplification and transformation *in vitro* as well as tumorigenesis *in vivo* (8, 9). Furthermore, the up-regulation of Aurora-A expression in diploid human breast epithelial cells leads to abnormal numbers of centrosomes and the induction of aneuploidy (8). A correlation between the up-regulated expression of Aurora-A and clinical aggressiveness has also been reported for several cancers (10–14). Moreover, recent reports showed that the up-regulation of Aurora-A resulted in resistance

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to apoptosis induced by taxanes in a human cancer cell line (15, 16) and inhibition of the expression of Aurora-A resulted in potent antitumor activity and chemosensitizing activity to taxanes in pancreatic cancer (17). These findings suggest that *Aurora-A* is a critical kinase-encoding gene and a potential chemotherapeutic target.

Esophageal squamous cell carcinoma (ESCC) is one of the most lethal malignancies in the world, including Japan, despite the recent refinement of various therapeutic strategies, including surgery, chemotherapy, radiotherapy, and combined therapy (18, 19). Docetaxel is used as monotherapy or in combination with other agents to treat ESCC, but its activity is far from satisfactory (20, 21). Therefore, identifying and targeting genes conducive to the treatment of ESCC, such as enhancement of conventional chemotherapy, is necessary to improve the survival of patients with this type of refractory cancer.

Using comparative genomic hybridization, we previously investigated changes in the copy number of chromosomes in 29 ESCC cell lines and found that a chromosome gain of the proximal part of 20q, where the *Aurora-A* gene is located, is one of the most common sites of aberrations (19 of 29, 65.5%; ref. 22). We also reported that the expression of Aurora-A was frequently up-regulated in ESCC tissues as well as cell lines, and this contributed to a poor prognosis (14). In the present study, to further elucidate the possibility of using *Aurora-A* in the treatment of human ESCC, we analyzed the phenotypic changes of cultured ESCC cells induced by suppression of Aurora-A expression using a plasmid vector-mediated short hairpin RNA (shRNA) expression system, especially synergistic enhancement of the cytotoxicity of docetaxel.

## Materials and Methods

**Cell culture.** All tested ESCC cell lines of the KYSE series were established in our laboratory and maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD) and Ham's F12 (Nissui Pharmaceutical, Tokyo, Japan) mixed (1:1) medium containing 2% fetal bovine serum (23). HeLa cells were purchased from the American Type Culture Collection (Rockville, MD), cultured in DMEM (Life Technologies) with 10% FCS, and used as a positive control (24, 25).

**Western blot analysis.** Cells were lysed in lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100] containing Complete Mini protease inhibitor (Roche Diagnostics, Mannheim, Germany). Cell lysates were sonicated and the protein concentration was estimated by the Bradford method using bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). Cell lysates (15  $\mu$ g) were electrophoresed on 2% to 15% gradient polyacrylamide gel (Daiichi Pure Chemicals, Tokyo, Japan) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) using a semidry transfer blot system (Bio-Rad, Hercules, CA). After blocking with TBS containing 1% Tween 20 and 5% skim milk for 1 h, the membranes were incubated at 4°C overnight with anti-human Aurora-A polyclonal antibody (diluted 1:100; TransGenic, Inc., Kumamoto, Japan) or anti-human  $\beta$ -actin monoclonal antibody (1:2,000; Sigma, Inc., St. Louis, MO). The membranes were subsequently incubated at room temperature for 1 h with secondary antibody and analyzed using enhanced chemiluminescence plus reagent (Amersham, Buckinghamshire, United Kingdom). Quantitative analysis was done on a Macintosh computer using the public domain NIH Image program version 1.61 (developed at the NIH and available on the Internet).<sup>3</sup>

**Immunofluorescent staining.** 510 A, 510 m, 1440 A, and 1440 m cells were cultured onto collagen-coated glass coverslips (BD Biosciences, Bedford, MA). Then, cells were washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The fixed cells were incubated with 0.3% Triton X-100 solution for 15 min at room temperature, and the cells were blocked for 1 h with 2% fetal bovine serum in PBS at room temperature. Subsequently, the cells were incubated with anti-human Aurora-A polyclonal antibody (diluted 1:50; TransGenic) for 1 h at room temperature. After washing twice with PBS, the cells were incubated with goat anti-rabbit FITC-conjugated secondary antibody (Invitrogen Co., Carlsbad, CA) for 1 h at room temperature. The cells were washed and mounted in glycerol and viewed under a laser scanning microscope (Axioskop 2 plus, Carl Zeiss Co. Ltd., Jena, Germany).

**Construction of an Aurora-A shRNA expression vector.** To construct a vector for Aurora-A shRNA, the pSUPERIOR-puro (OligoEngine, Seattle, WA) was digested with *Bgl*II and *Hind*III (TaKaRa Bio, Shiga, Japan). A chemically synthesized oligonucleotide encoding an Aurora-A short hairpin small interfering RNA, including a loop motif, was inserted downstream of the H1 promoter of the plasmid using a DNA ligation kit (TaKaRa Bio) and cloned. The sequence of the oligonucleotide targeted at Aurora-A is 5'-ATGCCCTGTCTACTGTCA-3' for KYSE 510, corresponding to positions 853 to 871 within the Aurora-A mRNA sequence (17). To confirm the result, we designed another sequence for KYSE 1440, 5'-GCCGGTTCAGAATCAGAAG-3', corresponding to positions 335 to 353 within the Aurora-A mRNA. For the negative control vector, an empty pSUPERIOR-puro was used. We checked the internal stabilities of each sequences using Oligo 4.0-s software (National Biosciences, Inc., Plymouth, MN) and found that the 3'-end in the sense strand was less stable than the 5'-end, which is known to cause less off-target effects according to literature (26). Furthermore, we checked each sequence using BLAST Web site software<sup>4</sup> and found that each sequence was specific to *Aurora-A*. Moreover, we also stably transfected each sequence to HeLa cell, and preliminary experiments were undertaken to further confirm their effects (Supplementary Data 1).

**Transfections.** The ESCC cell lines KYSE 510 and KYSE 1440 were stably transfected with the Aurora-A shRNA expression vector or the empty pSUPERIOR-puro vector using Lipofectin reagent (Invitrogen) as suggested by the manufacturer's instruction. Briefly, 2  $\mu$ g of each plasmid DNA and 20  $\mu$ L of Lipofectin reagent together with Opti-MEM I medium (Invitrogen) were used with serum-free medium for 8 h. Cells were incubated for another 48 h with normal growth medium, and subsequently, the cell clones were selected against 1.0  $\mu$ g/mL puromycin (Nacalai Tesque, Kyoto, Japan) for 3 weeks, and we picked up single colonies originated from single cells and expanded to obtain stably transfected cell lines.

**WST assay for sensitivity to docetaxel.** Cytotoxic activity against docetaxel was measured by the tetrazolium salt-based proliferation assay (WST-8 assay; Wako Chemicals, Osaka, Japan) following the manufacturer's instructions. Briefly, cells were cultured in 96-well microtiter plates in 90  $\mu$ L of growth medium (4,000 cells per well) and incubated for 24 h for sufficient cell growth. Then, 10  $\mu$ L of a graded concentration of docetaxel ( $10^{-5}$  to  $10^{-10}$  mol/L) were added into each well and cultured for 48 h. Control cultures received normal growth medium only. After 48 h, 10  $\mu$ L of WST-8 solution were added to each well and the plates were incubated at 37°C for another 3 h. Absorbance at 450 and 640 nm was measured using the Delta Soft ELISA analysis program, and cell viability was measured and compared with that of control cells. Each experiment was carried out independently and repeated at least thrice. The IC<sub>50</sub> value was defined as the concentration that reduced the absorbance in each test by 50%.

**Cell proliferation assay.** Cells were cultured in 6-cm dishes ( $2 \times 10^4$  per dish) and incubated for 24 h for sufficient cell growth and then treated with medium containing 0.3, 0.6, or 1.0 nmol/L of docetaxel for

<sup>3</sup> <http://rsb.info.nih.gov/niH-image/>

<sup>4</sup> <http://www.ddbj.nig.ac.jp/search/blast-j.html>

48 h. Then, they were cultured for another 48 h with normal growth medium. Control cultures received normal growth medium only. Cells were harvested with trypsin/EDTA every 48 h for 4 days and enumerated using a cell counter (Coulter Z1, Beckman Coulter, Fullerton, CA). A comparison was made with the control culture to examine the effect of suppressing the expression of Aurora-A on cell proliferation and to investigate the effect of the suppression on chemosensitivity to docetaxel. The experiment was repeated at least thrice.

**Flow cytometry for analyzing the cell cycle.** A flow cytometric analysis of DNA content was done to assess the cell cycle phase distribution. Cells were harvested at the 70% confluent stage and fixed in 70% ethanol at  $-20^{\circ}\text{C}$ . After being washed with PBS, the cells were treated with PBS containing RNase A (100 mg/mL) at  $37^{\circ}\text{C}$  for 30 min. After centrifugation, the cells were resuspended in PBS containing propidium iodide (50  $\mu\text{g}/\text{mL}$ ) and stained at room temperature for 30 min. DNA content was evaluated using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) and CellQuest software (Becton Dickinson). The experiment was repeated thrice.

**Flow cytometry for detection of apoptotic cells.** KYSE cells transfected with empty vector or shRNA against Aurora-A were cultured in 6-cm dishes ( $2 \times 10^4$  per dish) and treated as already described for the proliferation experiment. Briefly, cells were incubated for 24 h and treated with medium containing 0, 0.3, 0.6, or 1.0 nmol/L of docetaxel for 48 h. Then, they were cultured for another 48 h with normal growth medium. Subsequently, floating cells in the medium and adherent cells were collected. Using an Annexin V-FITC Apoptosis Detection kit (Medical & Biological Laboratories Co. Ltd., Woburn, MA), cells were stained with Annexin V-FITC and propidium iodide according to the manufacturer's instructions. Annexin V binds to cells that express phosphatidylserine on the outer layer of the cell membrane, and propidium iodide stains the cellular DNA of cells with a compromised cell membrane. This allows for the discrimination of live cells (unstained) from early apoptotic cells (stained only with Annexin V) and late apoptotic or necrotic cells (stained with both Annexin V and propidium iodide). Untreated cells and cells treated with 3% formaldehyde for 30 min served, respectively, as negative and positive controls for double staining. Cells were analyzed immediately after staining by using a FACScan flow cytometer and the CellQuest software. For each measurement,  $>10,000$  cells were counted.

**Tumor formation assay in nude mice.** Suspensions of  $1.0 \times 10^6$  KYSE 510-derived cells (Aurora-A shRNA-transfected cells, 510 A; empty vector-transfected cells, 510 m) in PBS (50  $\mu\text{L}$ ) were injected s.c. into the left flanks of 5-week-old male BALB/c *nu/nu* mice (Japan SLC, Shizuoka, Japan) at day 0. The inoculation was conducted in five mice, and tumor growth was estimated from the average volume of tumors. Tumor volume was calculated by the formula  $1/2 \times L^2 \times W$  ( $L$  = length and  $W$  = width of the tumor). At 46 days after inoculation, all mice were sacrificed, and s.c. tumors were resected and fixed in 10% formaldehyde/PBS. The tumors were paraffin embedded and stained with H&E and for Aurora-A. Immunohistochemical staining for Aurora-A was done as reported previously (14). All the animal experiments were done in accordance with institutional guidelines of the Kyoto University.

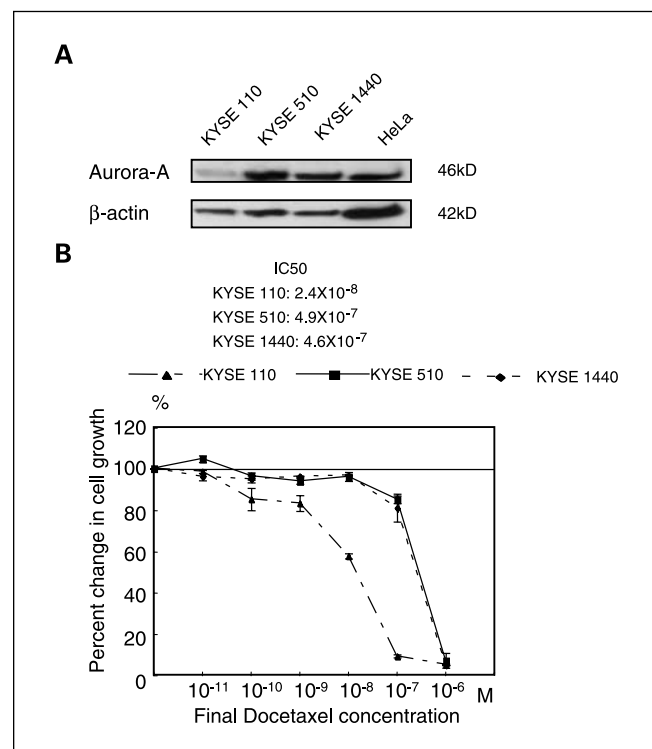
**Treatment with docetaxel in nude mice.** 510 m and 510 A tumors were generated as above. Briefly, suspensions of  $1.0 \times 10^6$  cells in PBS (50  $\mu\text{L}$ ) were injected s.c. into the left flanks of 5-week-old male BALB/c *nu/nu* mice at day 0. The inoculation was conducted in five mice, and mice were treated with docetaxel (7.0 mg/kg body weight; i.p., thrice weekly) or with 0.1 mL PBS (pH 7.4; i.p., thrice weekly) as described previously with modifications (27). Tumor growth was estimated from the average volume of tumors, and tumor volume was calculated as already described. At 39 days after inoculation, all mice were sacrificed, and s.c. tumors were resected, and tumor size was compared. All the animal experiments were done in accordance with institutional guidelines of the Kyoto University.

**Statistical analysis.** All experiments were done in duplicate or triplicate. The Bonferroni multiple comparison test and  $t$  test were used for the statistical analysis of comparative data using StatView version 5 (SAS Institute, Cary, NC). Values of  $P < 0.05$  were considered significant and are indicated by asterisks in the figures.

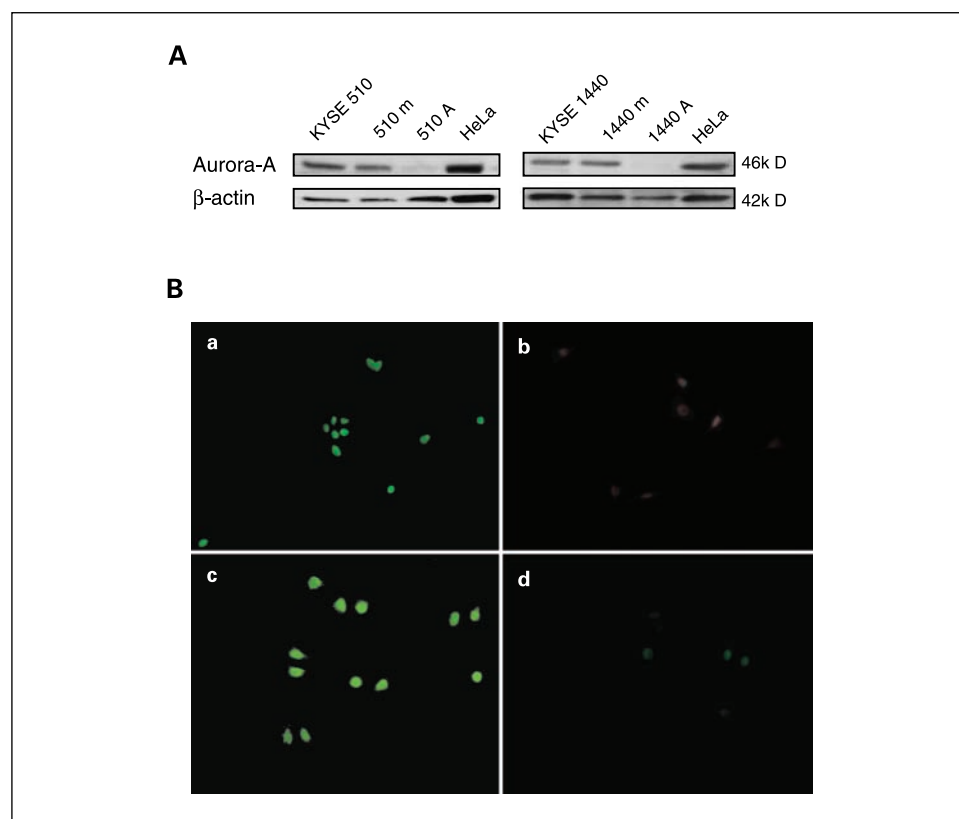
## Results

**Expression of Aurora-A and sensitivity to docetaxel in ESCC cell lines.** Levels of Aurora-A protein expression in three cancer cell lines were determined by Western blotting (Fig. 1A). KYSE 510 and KYSE 1440 had higher levels of expression than KYSE 110. Next, the sensitivity of the cell lines to docetaxel was checked by WST assay. As shown in Fig. 1B,  $\text{IC}_{50}$  of KYSE 510 and KYSE 1440 ( $4.9 \times 10^{-7} \pm 0.20 \times 10^{-7}$  mol/L and  $4.6 \times 10^{-7} \pm 0.46 \times 10^{-7}$  mol/L, respectively) was higher than that of KYSE 110 ( $2.4 \times 10^{-8} \pm 0.21 \times 10^{-8}$  mol/L; Fig. 1B). Thus, the cell lines with the higher levels of Aurora-A protein (KYSE 510 and KYSE 1440) were more resistant to docetaxel than the cell line with the lower level (KYSE 110).

**Vector-based Aurora-A shRNA decreased proliferation in ESCC cell lines.** To assess the role of the overexpression of Aurora-A in ESCC cells, we first established subclones via the transfection of vector-based shRNA for Aurora-A in KYSE 510 and KYSE



**Fig. 1.** Aurora-A expression and chemosensitivity to docetaxel in ESCC cell lines. **A**, expression of Aurora-A protein in ESCC cell lines. Immunoblots were probed with the anti-Aurora-A antibody and the anti- $\beta$ -actin antibody. The HeLa cell line was included as a positive control of Aurora-A expression. **B**, cytotoxic activities of docetaxel in ESCC cell lines were measured by WST-8 assay. Graded concentrations of docetaxel ( $10^{-5}$  to  $10^{-10}$  mol/L) were added to the wells, and the cells were cultured for 48 h. Control cultures received normal growth medium only. Cell viability was measured as absorbance at 450 nm, and values were compared with the control. The  $\text{IC}_{50}$  value was defined as the concentration that reduced the absorbance in each test by 50%. X axis, final docetaxel concentration ( $10^{-6}$  to  $10^{-11}$  mol/L). The experiments were repeated thrice.



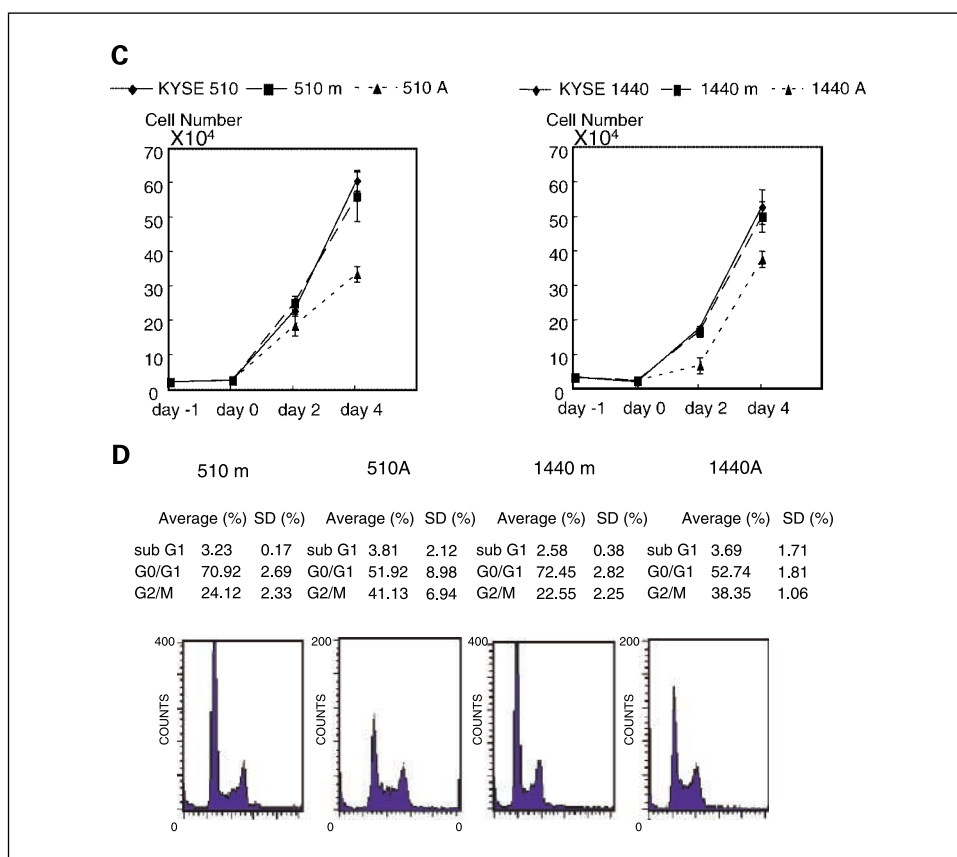
**Fig. 2.** Vector-based Aurora-A shRNA decreased proliferation of ESCC cell lines. **A**, suppression of Aurora-A protein expression with the Aurora-A shRNA vector. The KYSE 510 and KYSE 1440 cells were stably transfected with an empty vector (510 m or 1440 m) or an Aurora-A shRNA vector (510 A or 1440 A). The expression of Aurora-A protein in these clones was examined by Western blotting. Bottom, protein expression of β-actin. **B**, representative immunofluorescent staining of Aurora-A in an Aurora-A shRNA-transfected cells (510 A or 1440 A) or an empty vector-transfected cells (510 m or 1440 m). **a**, immunofluorescent staining of Aurora-A in empty vector-transfected cells (510 m). Magnification,  $\times 200$ . Strong nuclear staining was observed. **b**, immunofluorescent staining of Aurora-A shRNA-transfected cells (510 A). Magnification,  $\times 200$ . Weak nuclear staining was observed. **c**, immunofluorescent staining of Aurora-A in empty vector-transfected cells (1440 m). Magnification,  $\times 200$ . Strong nuclear staining was observed. **d**, immunofluorescent staining of Aurora-A in Aurora-A shRNA-transfected cells (1440 A). Magnification,  $\times 200$ . Weak nuclear staining was observed.

1440 cells. The levels of Aurora-A expression were efficiently reduced by 87% in the stable subclone 510 A and by 90% in 1440 A but were not reduced in empty vector-transfected clones (510 m and 1440 m; Fig. 2A). We also confirmed suppression of Aurora-A expression by immunofluorescent staining and found that Aurora-A immunoreactivity in established stable subclone 510 A and 1440 A was homogeneously depleted (Fig. 2B). We investigated the effect of suppressing the expression of Aurora-A on proliferation in ESCC cell lines. With suppression of Aurora-A expression, cell growth was inhibited by  $38.7 \pm 10.5\%$  in 510 A and by  $24.3 \pm 10.0\%$  in 1440 A compared with empty vector-transfected cells (510 m and 1440 m;  $P < 0.01$  and  $0.01$ , respectively; Fig. 2C). To investigate the growth suppression caused by inhibition of Aurora-A expression, cell cycle changes in 510 A and 1440 A as well as in empty vector-transfected cells were examined by flow cytometry. The population of cells in G<sub>2</sub>-M phase was significantly larger in 510 A ( $41.13 \pm 6.94\%$ ) and 1440 A ( $38.35 \pm 1.06\%$ ) than that in the empty vector-transfected cells (510 m:  $24.12 \pm 2.33\%$ ,  $P < 0.01$ ; 1440 m:  $22.55 \pm 2.25\%$ ,  $P < 0.01$ ; Fig. 2D).

**The effect of suppressing Aurora-A on chemosensitivity to docetaxel in vitro.** Chemosensitivity to docetaxel was investigated by WST assay and cell number determinations. In the WST assay, we found that IC<sub>50</sub> of stable transfectants of shRNA for Aurora-A was lower than that of the empty vector transfectant (510 A,  $2.7 \times 10^{-7} \pm 0.4 \times 10^{-7}$  mol/L; 510 m,  $4.8 \times 10^{-7} \pm 0.7 \times 10^{-7}$  mol/L; 1440 A,  $2.6 \times 10^{-7} \pm 0.8 \times 10^{-7}$  mol/L; 1440 m,  $4.9 \times 10^{-7} \pm 0.4 \times 10^{-7}$  mol/L; Fig. 3A and B). We also confirmed the effect of suppressing Aurora-A

expression on chemosensitivity to docetaxel by cell number determinations. In the cell number determinations, the suppression of the expression of Aurora-A allowed even 0.3 nmol/L docetaxel to be effective (Fig. 3C and D). Discrepancy in effective concentration of docetaxel between WST assay and cell number determinations could be explained by the difference in the initial numbers of cells, cell concentration at the beginning of each experiment, as well as the difference in size of culture plates and observation period. We repeated the cell number determinations with the same cell concentration and observation period as WST assay and confirmed that growth-inhibitory effect of docetaxel in these two different experiments was similar (Supplementary Data 2). Importantly, in respective experiments, we found that the suppression of Aurora-A expression enhanced chemosensitivity to docetaxel. To investigate the effect of chemosensitivity to docetaxel by the suppression of Aurora-A expression, apoptotic cells in the 510 A and 1440 A clones as well as empty vector-transfected clones were examined by flow cytometry and the type of cell death induced by docetaxel was assessed. In the Aurora-A shRNA-transfected cells, 0.3 nmol/L docetaxel induced a notable level of apoptosis compared with the empty vector-transfected cells (510 A, 71.3%; 510 m, 36.12%; 1440 A, 78.1%; 1440 m, 26.28%; Fig. 3E and F). These findings suggested that the suppression of Aurora-A expression augmented the apoptosis induced by docetaxel. To confirm the validity of the experiment, cells treated with 3% formalin for 30 min and stained with both Annexin V and propidium iodide served as a positive control for double staining (Supplementary Data 3).

**Fig. 2** Continued. *C*, *in vitro* growth assay in cells transfected with the Aurora-A shRNA vector (510 A or 1440 A) or an empty vector (510 m or 1440 m). The number of cells was counted every 2 d. The experiments were repeated thrice. *D*, cell cycle profiles obtained by flow cytometry. Ratios of cell populations in G<sub>1</sub> and G<sub>2</sub>-M were the average and SD. These experiments were done thrice, and representative results are shown. The experiments were repeated thrice, and the representative results are shown.



**The effect of Aurora-A suppression on tumor growth *in vivo*.** To examine the possible effect of Aurora-A shRNA on tumor growth *in vivo*, s.c. tumor formation assay in nude mice was done. As shown in Fig. 4A, the tumors formed from Aurora-A shRNA-transfected cells (510 A) were clearly smaller than that formed from empty vector-transfected cells (510 m). Then, we examined tumor volume and weight at 46 days after inoculation. The average tumor volume of the mice at day 46 was reduced by 36.1% in 510 A compared with 510 m ( $P = 0.03$ ; Fig. 4C), and the average tumor weight was also decreased by 31.5% in 510 A compared with 510 m ( $P = 0.03$ ; Fig. 4C). All of the tumors were stained with H&E and for Aurora-A (Fig. 4D), and Aurora-A immunoreactivity was confirmed to be reduced in tumors grown from Aurora-A shRNA-transfected cells but not in tumors grown from empty vector-transfected cells.

**The effect of Aurora-A suppression on chemosensitivity to docetaxel *in vivo*.** To examine the possible effect of Aurora-A shRNA on chemosensitivity to docetaxel *in vivo*, s.c. tumors were generated in nude mice followed by treatment with docetaxel or PBS. The tumors formed from Aurora-A shRNA-transfected cells (510 A) were apparently smaller than those formed from empty vector-transfected cells (510 m) after the treatment with docetaxel at day 39 (Fig. 5A, b and d). To confirm this, we measured tumor volume at 39 days after inoculation. As shown in Fig. 5C, following the treatment with docetaxel, the average tumor volume of 510 A and 510 m was  $175.3 \pm 70.4 \text{ mm}^3$  and  $312.8 \pm 28.0 \text{ mm}^3$ , respectively, and thus, the suppression of Aurora-

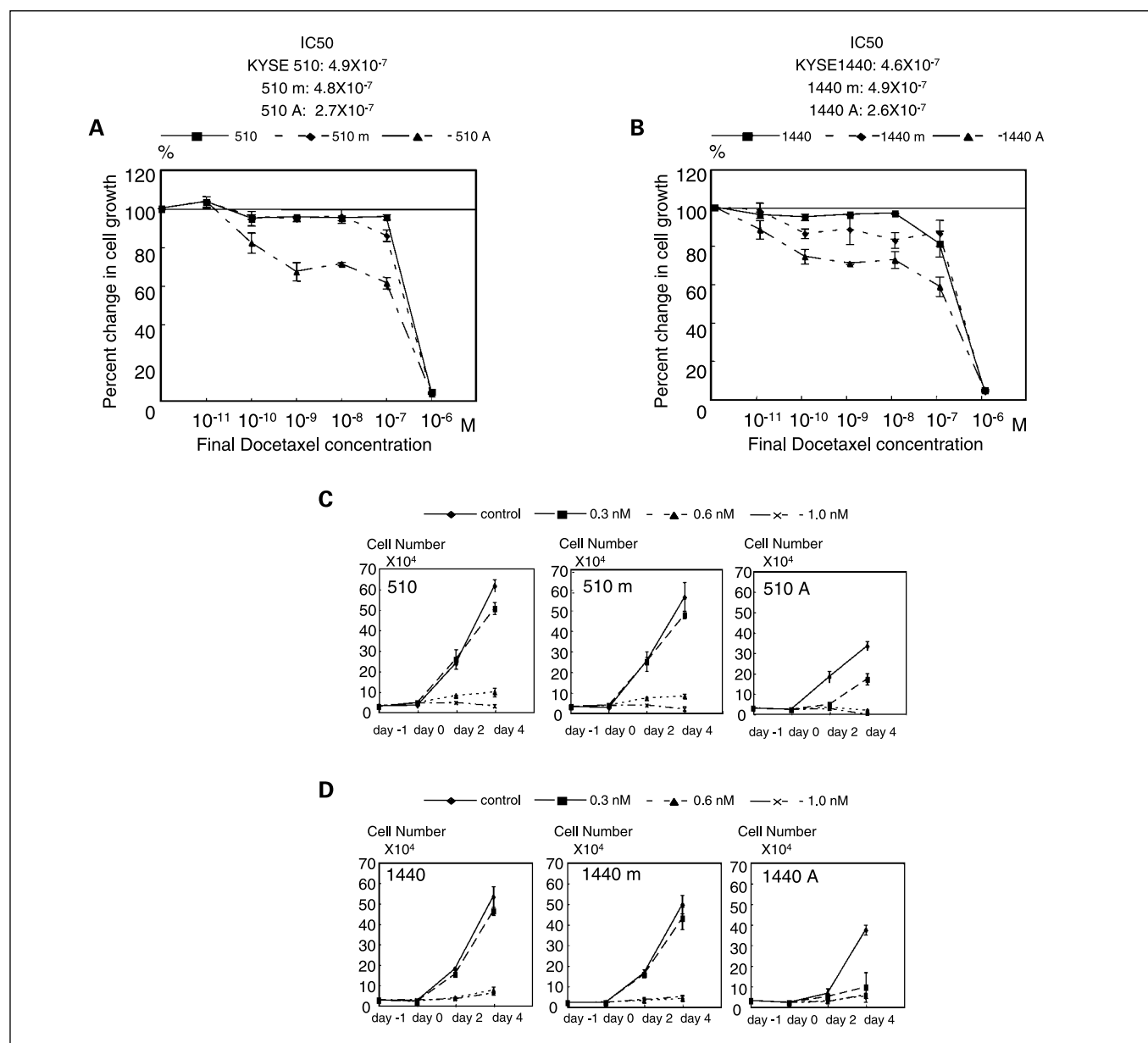
A expression resulted in a 44.0% inhibition of tumor growth ( $P = 0.03$ ).

## Discussion

We previously reported that the expression of Aurora-A was frequently up-regulated in ESCC tissues and cell lines and contributed to a poor prognosis (14). In an attempt to determine the potential of Aurora-A as a therapeutic target, we used a vector-based shRNA technique to knock out its expression and analyzed its phenotypes.

In the current study, we were able to suppress the expression of Aurora-A using vector-based shRNA for two different target sequences in two different ESCC cell lines and obtained very similar results at each examination *in vitro*. That is, a reduction of Aurora-A protein expression was clearly related to cell growth inhibition and increased sensitivity to docetaxel.

Recently, a relationship between Aurora-A activity and G<sub>2</sub>-M transition was reported in cancer cells (28). In the current study, we showed that the suppression of Aurora-A expression caused an accumulation of the cells in the G<sub>2</sub>-M phase *in vitro*, resulting in the inhibition of proliferation of ESCC cell lines. Moreover, in recent report, Hata et al. (17) showed that the suppression of Aurora-A expression had an antitumorigenic effect *in vivo* in pancreatic cancer, and we similarly showed that the suppression of Aurora-A expression in ESCC cell lines inhibited tumor growth *in vivo* using our vector-mediated shRNA strategy. Our results were consistent with Hata et al. in



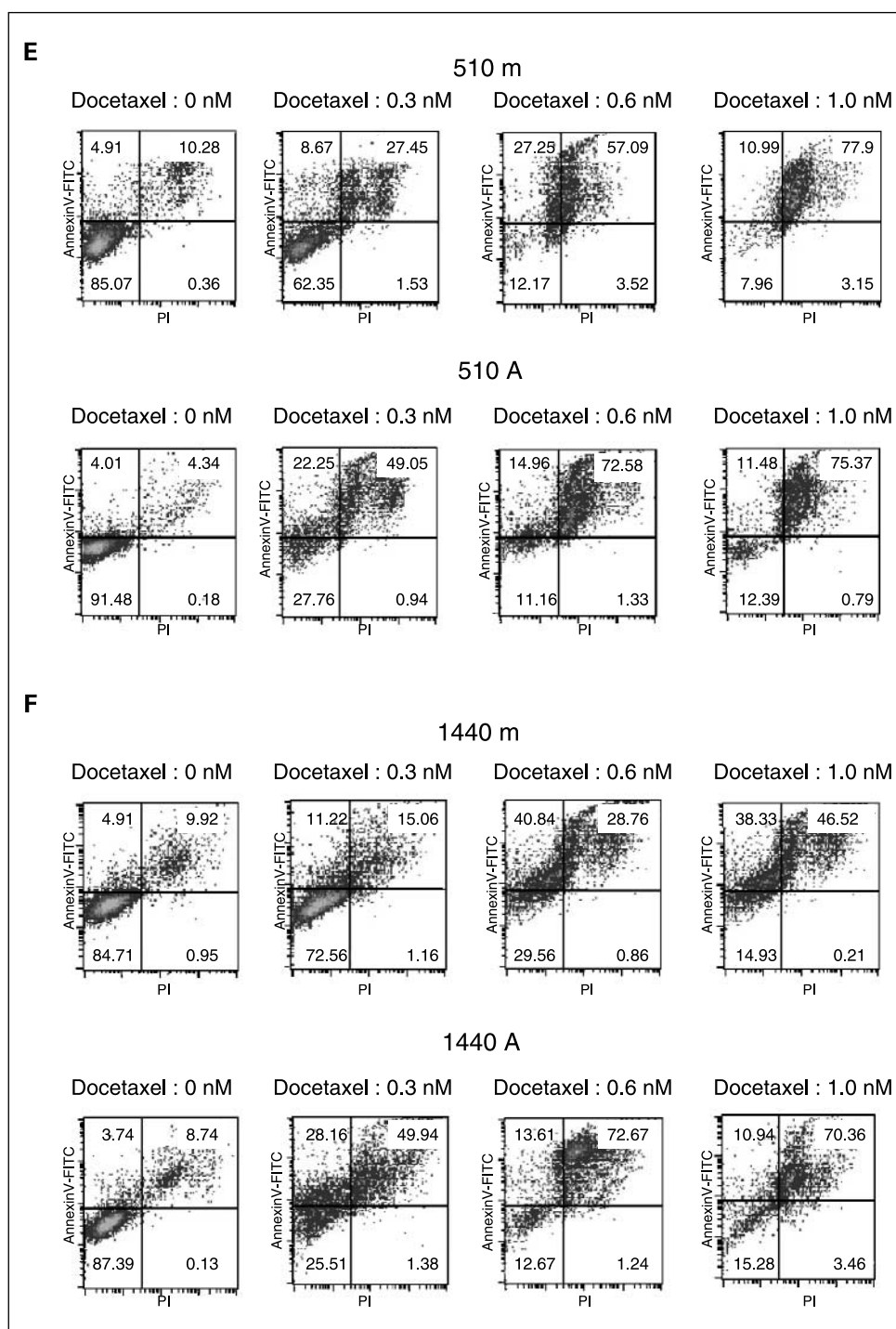
**Fig. 3.** Effect of the suppression of Aurora-A expression on sensitivity to docetaxel. *A* and *B*, cytotoxic activities of docetaxel were measured by WST-8 assay. Graded concentrations of docetaxel ( $10^{-5}$  to  $10^{-10}$  mol/L) were added to the wells, and the cells were cultured for 48 h. Control cultures received normal growth medium only. Cell viability was measured as absorbance at 450 nm, and values obtained were compared with that of the control. The  $IC_{50}$  value was defined as the concentration that reduced the absorbance in each test by 50%. X axis, final docetaxel concentration ( $10^{-6}$  to  $10^{-11}$  mol/L). The experiments were repeated thrice. *C* and *D*, cytotoxic activities of docetaxel were confirmed by cell counting. Graded concentrations of docetaxel (0, 0.3, 0.6, and 1.0 nmol/L) were added for the first 2 d, and normal growth medium was added for the next 2 d. The number of cells was counted every 2 d. These experiments were repeated thrice.

terms of antitumor activity that the suppression of Aurora-A expression caused in ESCC. Consequently, Aurora-A seemed to be a critical factor for the proliferation of cancer cells, and therefore, it should be a good therapeutic target for halting proliferation of ESCC.

Furthermore, we found that the suppression of Aurora-A expression enhanced the sensitivity to docetaxel-induced apoptosis both *in vitro* and *in vivo*. Taxanes bind to free tubulin and promote the assembly of tubulin into stable microtubules. Hence, they stop cell cycle progression, causing cells in the M phase to accumulate at the metaphase-anaphase transition and

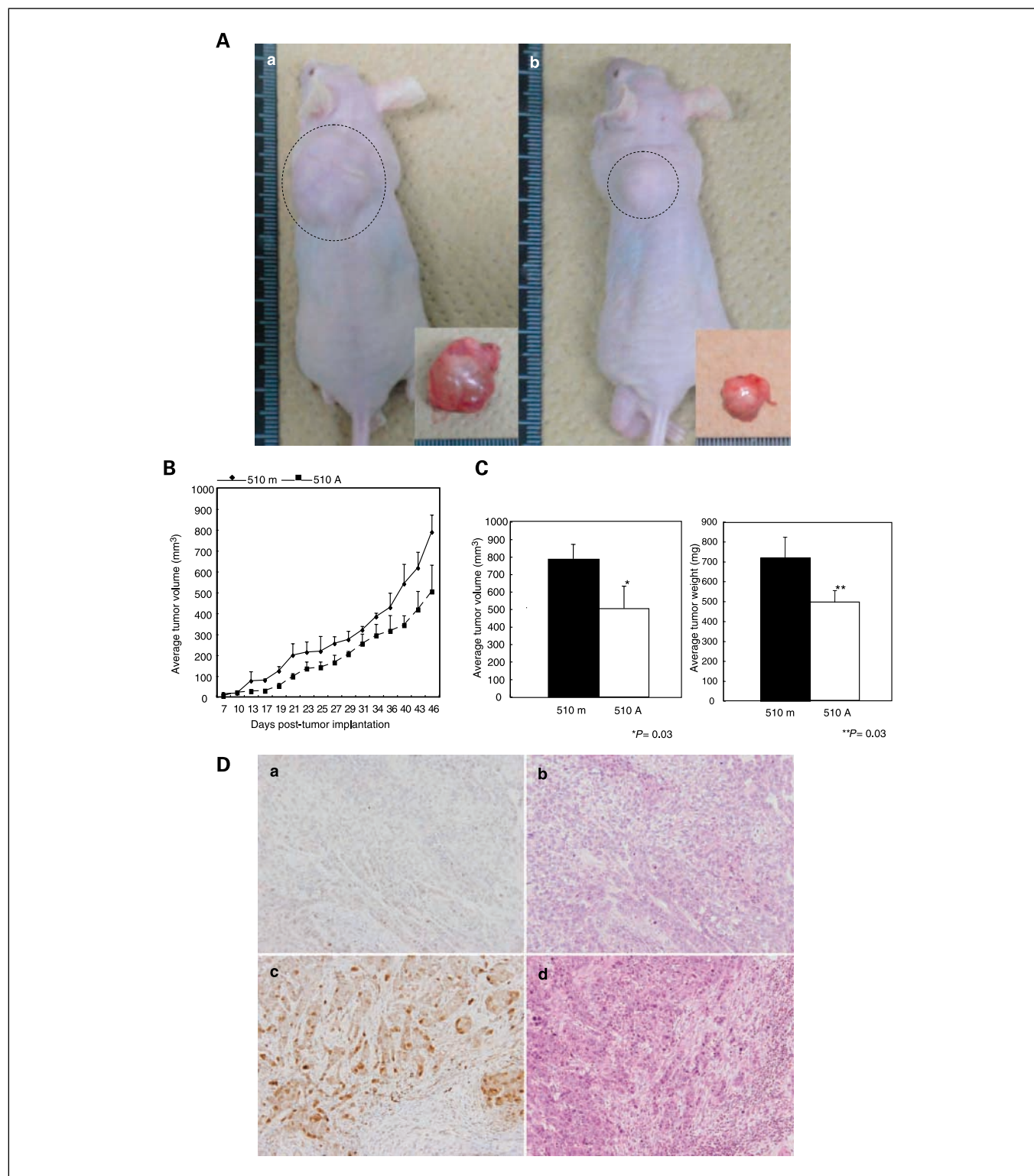
subsequently leading them to apoptosis, which is consistent with our findings that the suppression of Aurora-A expression resulted in the accumulation of cells in the  $G_2$ -M phase. As previously discussed, Aurora-A is essential for the proper arrangement of centrosomes and microtubules, and Hata et al. showed that a combination of the suppression of Aurora-A expression and use of taxanes resulted in an augmented induction of apoptosis in pancreas cancer *in vitro* (17). Additionally, Anand et al. (15) showed that the over-expression of Aurora-A induced increased resistance to taxanes via a decrease in spindle checkpoint activity *in vitro*. Our results

**Fig. 3 Continued.** E and F, to assess the type of cell death induced by docetaxel, flow cytometry was done. Cells in bottom left quadrant (unstained), top left quadrant (stained only with Annexin V), and top right quadrant [stained with both Annexin V and propidium iodide (PI)] represent viable cells, cells in early apoptosis, and cells in late apoptosis, respectively. The experiments were repeated thrice, and the representative results are shown.



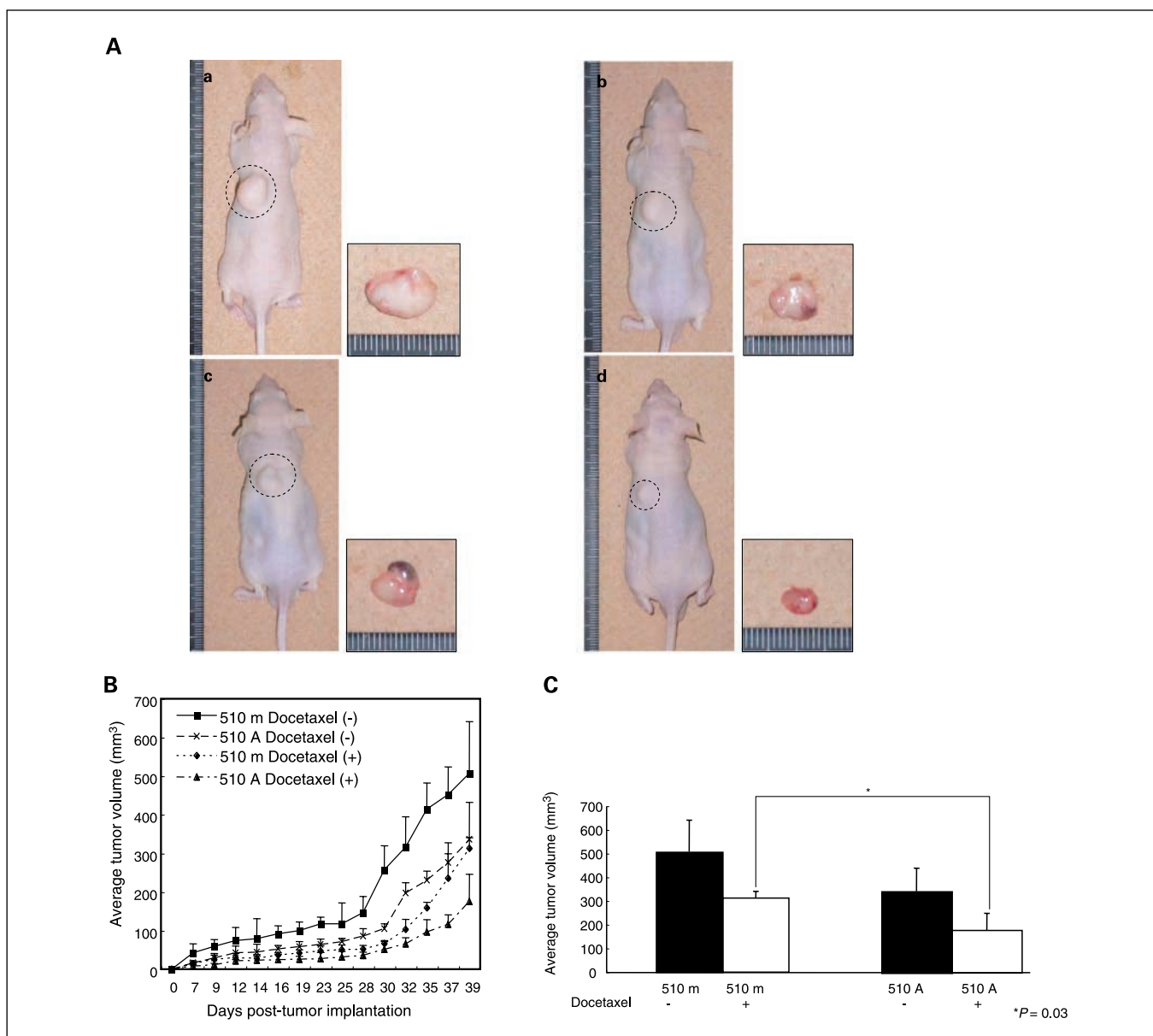
are consistent with this study and were able to show that the suppression of Aurora-A expression enhanced chemosensitivity to docetaxel not only *in vitro* but also *in vivo*. These results suggest that, by suppressing Aurora-A expression, spindle checkpoint activity might have recovered and, thus, increased the sensitivity to taxanes. In the meantime, the mechanism that triggers apoptosis after inhibition of Aurora-A expression, as well as the complicated biological activity of Aurora-A, remains to be clarified.

RNA interference has become conventional applications for *in vivo* cancer therapy (29, 30), and an efficient way of delivering small interfering RNA into solid tumors has been developed (31). In the current study, we explored the possibility that the RNA interference-mediated suppression of Aurora-A could be used as a specific gene-targeting therapy to suppress the progression of ESCC. Moreover, the function of Aurora kinase inhibitors (including the patent literature) has been studied recently, revealing potentially promising anticancer



**Fig. 4.** Effect of the down-regulation of Aurora-A expression on the formation of tumors *in vivo*. **A**, representative features of tumors in a mouse 46 d after the inoculation. **a**, tumors formed from empty vector – transfected cells (510 m) in the left flank; **b**, tumors formed from Aurora-A shRNA-transfected cells (510 A) in the left flank. **B**, growth of tumors in the mice injected with Aurora-A shRNA-transfected cells (510 A) or empty vector – transfected cells (510 m). The inoculation was done in five mice. **C**, tumor volume and weight at day 46 after inoculation. Left, black column, average tumor volume at day 46 after the inoculation of empty vector – transfected cells (510 m); white column, average tumor volume at day 46 after the inoculation of Aurora-A shRNA-transfected cells (510 A;  $n = 5$ ). \*,  $P = 0.03$ . Right, black column, average tumor weight at day 46 after the inoculation of Aurora-A shRNA-transfected cells (510 A;  $n = 5$ ). \*\*,  $P = 0.03$ . **D**, **a**, immunohistochemical staining of Aurora-A in s.c. tumors at day 46 after the inoculation of Aurora-A shRNA-transfected cells (510 A). Magnification,  $\times 200$ . **b**, H&E staining of s.c. tumors at day 46 after the inoculation of Aurora-A shRNA-transfected cells (510 A). Magnification,  $\times 200$ . **c**, immunohistochemical staining of Aurora-A in s.c. tumors at day 46 after the inoculation of empty vector – transfected cells (510 m). Magnification,  $\times 200$ . **d**, H&E staining of s.c. tumors at day 46 after the inoculation of empty vector – transfected cells (510 m). Magnification,  $\times 200$ .





**Fig. 5.** Effect of the suppression of Aurora-A expression on sensitivity to docetaxel *in vivo*. Mice were treated with docetaxel (7.0 mg/kg body weight; i.p., thrice weekly) or with 0.1 mL PBS (pH 7.4; i.p., thrice weekly). **A**, representative features of tumors 39 d after inoculation. **a**, empty vector – transfected cells (510 m) were injected s.c. into the left flank and the mice were treated with PBS (i.p., thrice weekly). **b**, empty vector – transfected cells (510 m) were injected s.c. into the left flank and the mice were treated with docetaxel (7.0 mg/kg body weight; i.p., thrice weekly). **c**, Aurora-A shRNA-transfected cells (510 A) were injected s.c. into the left flank and the mice were treated with PBS (i.p., thrice weekly). **d**, Aurora-A shRNA-transfected cells (510 A) were injected s.c. into the left flank and the mice were treated with docetaxel (7.0 mg/kg body weight; i.p., thrice weekly). **B**, growth of tumors in the mice injected with Aurora-A shRNA-transfected cells (510 A) or empty vector – transfected cells (510 m) with or without docetaxel. The inoculation was done in five mice. **C**, tumor volume at day 39 after the inoculation. Left, black column, average tumor volume at day 39 after the inoculation of empty vector – transfected cells (510 m) in mice treated with PBS; white column, average tumor volume at day 39 after the inoculation of empty vector – transfected cells (510 m) in mice treated with docetaxel ( $n = 5$ ). Right, black column, average tumor volume at day 39 after the inoculation of Aurora-A shRNA-transfected cells (510 A) in mice treated with PBS; white column, average tumor volume at day 39 after the inoculation of Aurora-A shRNA-transfected cells (510 A) in mice treated with docetaxel ( $n = 5$ ). \*,  $P = 0.03$ .

effects (32, 33). Therefore, our results in combination with these findings suggest that taxane-mediated chemotherapy could be more effective in combination with these anti-Aurora agents in ESCC.

In summary, the suppression of Aurora-A expression is shown to inhibit tumor growth of ESCC and enhanced chemosensitivity to docetaxel both *in vitro* and *in vivo*. Consequently, the therapeutic regimen to suppress the Aurora-A expression is a

feasible candidate to become a novel therapeutic strategy for the treatment of ESCC.

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