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Curcumin-Induced Mitotic Spindle Defect and Cell Cycle Arrest in Human Bladder Cancer Cells Occurs Partly through Inhibition of Aurora A[S]

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ABSTRACT

Curcumin, an active compound in turmeric and curry, has been proven to induce tumor apoptosis and inhibit tumor proliferation, invasion, angiogenesis, and metastasis via modulating numerous targets in various types of cancer cells. Aurora A is a mitosis-related serine-threonine kinase and plays important roles in diverse human cancers. However, the effect of curcumin on Aurora A has not been reported. In this study, Aurora A promoter activity and mRNA expression were inhibited in curcumin-treated human bladder cancer T24 cells, suggesting that Aurora A is regulated at the transcription level. We also found that curcumin preferentially inhibited the growth of T24 cells, which show a higher proliferation rate, invasion activity, and expression level of Aurora A compared with that of human immortalized uroepithelial E7cells. Furthermore, inhibition of phosphorylation of Aurora A and its downstream target histone H3 accompanied by the formation of monopolar spindle, induction of G_2/M phase arrest, and reduction in cell division in response to curcumin were detected in T24 cells. These curcumin-induced phenomena were similar to those using Aurora A small interfering RNA and were attenuated by ectopic expression of Aurora A. Therefore, the antitumor mechanism of curcumin is Aurora A-related, which further supports the application of curcumin in treatments of human cancers.

Introduction

Curcumin, a major yellow pigment and spice in turmeric and curry, exhibits anticarcinogenic effects (Aggarwal and Shishodia, 2006). The therapeutic values of curcumin have been proven in human clinical studies (Anand et al., 2008). More importantly, curcumin is safe in humans even at a dose of 10 g/day (Goel et al., 2008). The ability of curcumin to interact with multiple target molecules and modulate multiple cellular signaling pathways may be the key to its therapeutic potential against cancers (Anand et al., 2008), because cancer growth and progression is a complex process that involves multiple signaling pathways (Anand et al., 2008). Possible anticancer mechanisms of curcumin include modulation of the cell cycle (Chen and Huang, 1998) and induction of apoptosis (Woo et al., 2003).

Bladder cancer is the second most common genitourinary malignancy in the United States (Jemal et al., 2010). Although systemic chemotherapy has improved the duration and the quality of life of patients, the long-term survival rates are poor (Gallagher et al., 2008). Improvement in chemotherapeutic regimens is urgently needed. It is noteworthy that suppression in proliferation of bladder cancer cells by curcumin has been observed in culture cells and animals (Anand et al., 2008). A phase I clinical trial in patients with resected bladder cancer also indicates the efficacy and pharmacological safety of curcumin (Goel et al., 2008).

Aurora A is an oncoprotein. The biological functions of Aurora A include regulation of centrosomal and microtubule activity and control of chromosome segregation (Giet et al., 2005). Overexpression of Aurora A in bladder cancer cells has been observed (Sen et al., 2002; Fraizer et al., 2004). A

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ABBREVIATIONS: DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; MTT, 3-[4,5]-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT, reverse transcription; PCR, polymerase chain reaction; siRNA, small interfering RNA; PI, propidium iodide.

retrospective cohort study of 205 patients with urinary bladder carcinoma also reveals a strong association between Aurora A expression and clinical aggressiveness of bladder cancer (Sen et al., 2002). Elevated chromosomal instability in bladder cancer cells leading to aneuploidy by overexpression of Aurora A has been suggested (Fraizer et al., 2004). Aurora A has been considered as a molecular target for cancer therapy. Molecules that inhibit Aurora A and exhibit anticancer activity in preclinical studies are currently under clinical evaluation (Karthigeyan et al., 2010).

In the present study, the association of Aurora A kinase with the curcumin-induced anticancer mechanism was investigated in Aurora A-overexpressing bladder cancer cells (Lin et al., 2006). Overall, our results showed that curcumin significantly inhibited Aurora A gene expression and subsequently kinase activity, which at least in part caused failure of various mitotic events and G_2/M mitotic arrest of human bladder cancer cells. The safety, low cost, and efficacy of curcumin may make it a promising agent for treatment of human bladder cancer.

Materials and Methods

Cell Culture and Curcumin Treatment. Immortalized human uroepithelial cell line E7 and grade III human bladder cancer T24 cells (American Type Culture Collection, Manassas, VA) were cultured with complete Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Invitrogen) in a humidified atmosphere of a 5% CO_2 incubator at 37°C. Curcumin (Sigma-Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide (Sigma-Aldrich). Control cells were cultured in medium containing an equal amount of dimethyl sulfoxide without curcumin.

Transient Transfection. The empty vector (pCMV2-flag) or FLAG-tagged wild-type Aurora A (pCMV2-flag-Aurora A) (Yu et al., 2005) was transfected using Lipofectamine 2000 (Invitrogen) in DMEM without supplementation of FBS according to the manufacturer's instructions.

Cell Growth Assay. Cell growth was determined using a 3-[4,5]-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) modified colorimetric assay (Lee et al., 2005). After treatment, MTT was added to each well. The absorbance of each well was determined at 590 nm in an enzyme-linked immunosorbent assay reader (MRX II; Thermo Fisher Scientific, Waltham, MA).

Cell Invasion Assay. Matrigel (BD Biosciences, Bedford, MA) in phosphate buffer solution (Merck, Darmstadt, Germany) was used for invasion assay. Cells suspended with DMEM without FBS were seeded on the upper well of a Transwell (Corning Incorporated, Lowell, MA). The lower well contained complete DMEM. After an 18-h incubation, the cells on the lower well stained with 0.1% of crystal violet (Merck) were counted under a light microscope (Olympus, Tokyo, Japan).

Western Blot Analysis. After treatment, the whole-cell lysates were subjected to Western blot analysis (Lee et al., 2005) using rabbit polyclonal anti-Aurora A (Cell Signaling Technology, Danvers, MA), mouse monoclonal anti-phospho-histone H3 (Ser10) (Cell Signaling Technology), mouse monoclonal anti-β-actin (Sigma-Aldrich), or rabbit polyclonal anti-phospho-Aurora A (Thr288) (BioLegend, San Diego, CA). After washing, a secondary probe of goat anti-rabbit or goat anti-mouse conjugated horseradish peroxidase secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) was detected directly with a Biospectrum Imaging System (UVP, Inc., Upland, CA).

RT-PCR. One microgram of the total RNA was reversed-transcribed according to the manufacturer's instruction (Promega, Madison, WI). The sequences of forward and reverse primers (Genomics Bioscience and Technology, Taipei, Taiwan) to amplify Aurora A were 5′-GAAATTGGTCGCCCTC-3′ and 5′-TGATGAATTTGCTGTGATCCA-3′, respectively. PCRs were performed in a PCR machine (TP600; TaKaRa, Shinga, Japan) programmed to predenature at 94°C for 1 min, followed by 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min for a total of 32 cycles. After the last cycle, the mixtures were incubated at 72°C for 7 min; 18S served as a control. The sequences of forward and reverse primers (Genomics Bioscience and Technology) to amplify 18S RNA were 5′-AAACGGCTACCACATCCAAG-3′ and 5′-CCTCCAATGGATCCTCGTTA-3′, respectively.

Luciferase Assay. According to the manufacturer's instruction (luciferase assay kit; Promega), the promoter activity of Aurora A was analyzed after cotransfection of 1 μg of pGL2-AAP plasmid (Aurora A promoter with luciferase reporter) (Hung et al., 2008) together with 0.25 μg of pRL-TK plasmid (*Renilla* luciferase reporter; Promega) (Farr and Roman, 1992) as a transfection efficiency control (Hung et al., 2008). The luciferase and *Renilla* measurement were performed using a luciferase assay system (Dual-Glo; Promega).

Small Interfering RNA and Transfection. Aurora A siRNA (sense 5′-GCAGAGAACUGCUACUUAU-3′ and antisense 5′-AUAAGUAGCAGUUCUCUGC-3′) was obtained from MDBio (Taipei, Taiwan). Transfections were performed using siRNA (250 nM) and Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). c-Met siRNA (sense 5′-CACGGCUCUAGCUGUCGAC-3′ and antisense 5′-GUCGACAACUAGAGCCAUG-3′) obtained from QIAGEN (Taipei, Taiwan) was used as a control.

Cell Cycle Determination. After treatment, cells fixed in 70% ethanol (Sigma-Aldrich) were incubated with 40 μg/ml propidium iodide (PI) (Sigma-Aldrich) and 0.25 μg/ml RNase A (AMRESCO, Solon, OH). The PI-stained cells were sorted in a FACScan flow cytometer (BD, San Jose, CA) (Lee et al., 2005).

Microscopy. After fixing and permeabilization, the cells were stained with rabbit monoclonal anti-α-tubulin antibody (Sigma-Aldrich), rabbit polyclonal anti-γ-tubulin antibody (Sigma-Aldrich), or mouse monoclonal anti-Aurora A antibody (Novocastra, Bannockburn, IL) (Lee et al., 2005). After washing, the cells were stained with fluorescein isothiocyanate-conjugated AffiniPure donkey anti-mouse IgG or goat-anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Nuclei were visualized by incubating the cells with PI (5 μg/ml). The signal was examined by using a fluorescence microscope (Leica DMRBE microscope) or a Leica TCSNT laser scanning confocal imaging system coupled to a Leica DMRBE microscope.

Statistics. Results are expressed as means ± S.E.M.. The data were analyzed by one-way analysis of variance. Differences among groups were analyzed by Duncan's multiple range test (SPSS software, version 14.0; SPSS, Chicago, IL). A difference was considered if $p < 0.05$.

Results

Curcumin Suppressed Aurora A mRNA Expression and Promoter Activity. To identify genes involved in the anticancer mechanism of curcumin, we had performed a cDNA microarray analysis and discovered that curcumin significantly inhibited Aurora A mRNA expression (S.-J. Won, J.-W. Shin, H.-S. Liu, and C.-L. Su, unpublished data). Although the link between curcumin and Aurora A has not been reported in the literature, both curcumin (Meeran and Katiyar, 2008) and Aurora A (Marumoto et al., 2005) regulate the cell cycle. In addition, administration of curcumin (Woo et al., 2003) or inhibition of Aurora A by siRNA (Du and Hannon, 2004) induces apoptosis. Therefore, we hypothesized that the anticancer activity of curcumin is Aurora A-related.

To determine at what level Aurora A was regulated by curcumin, Aurora A mRNA expression and promoter activity

were analyzed. Consistent with the result of cDNA microarray analysis, a significant decrease ($p < 0.01$) in Aurora A mRNA expression was detected by RT-PCR when the cells were treated with 30 μM curcumin for 48 h (Fig. 1A). Furthermore, Aurora A promoter activity was measured under the same conditions. As shown in Fig. 1B, curcumin inhibited

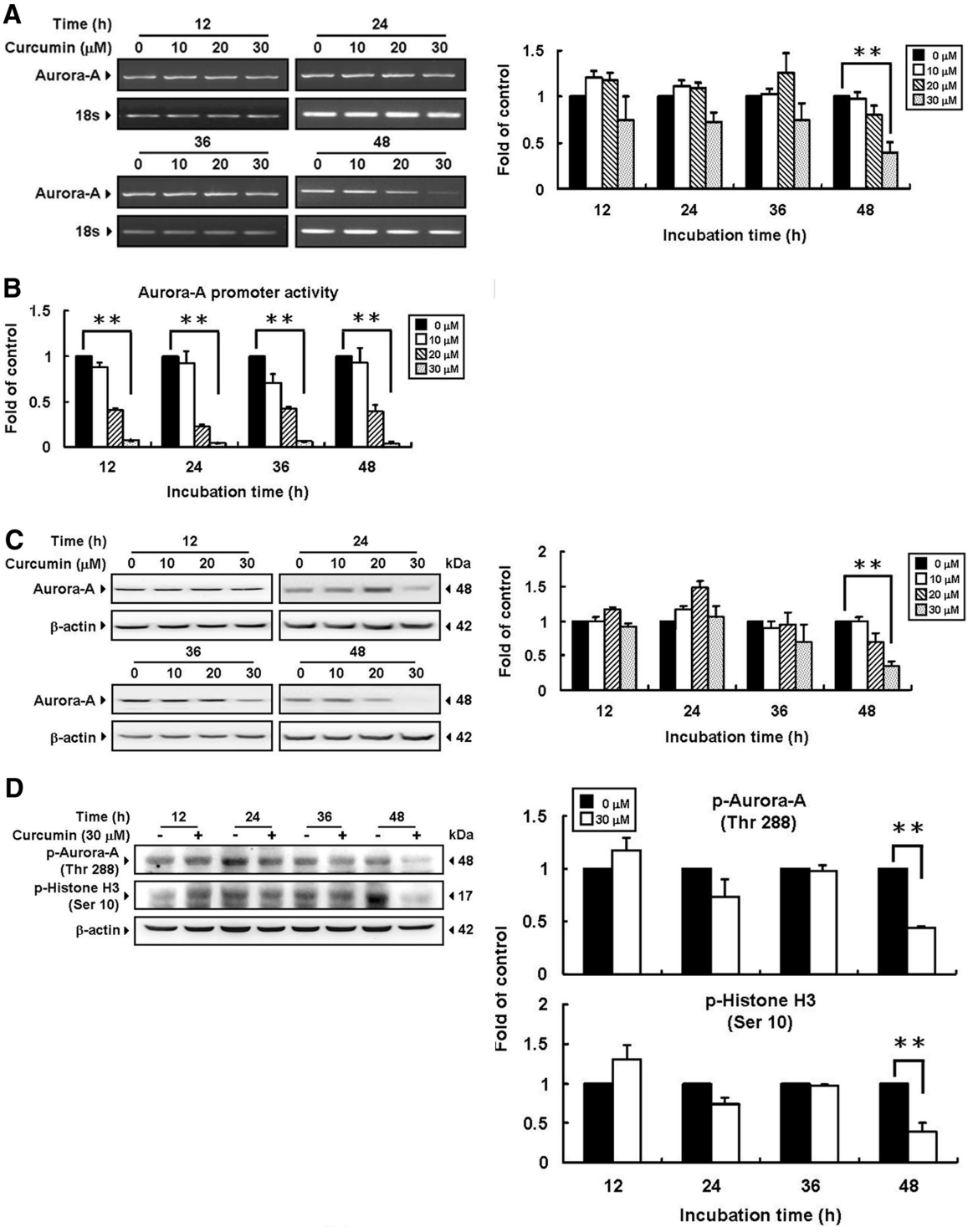

Fig. 1. Changes of Aurora A in T24 cells in response to curcumin. A, curcumin decreased Aurora A mRNA expression. Extracted total RNA (1 μg) was subjected to RT-PCR analysis. The intensity of individual Aurora A signal was quantified by densitometry normalizing to that of 18S RNA, with the control level arbitrarily set to 1. B, curcumin inhibited Aurora A promoter activity. Cells were cotransfected with pGL2-AAP together with pRL-TK for 4 h before exposure to curcumin. Aurora A luciferase activity was normalized to that of *Renilla*, with control level arbitrarily set to 1. C, curcumin suppressed Aurora A protein expression. After treatment, total protein was subjected to Western blot analysis. Anti-Aurora A antibody served as a probe. β-Actin served as a loading control. The intensity of each protein expression band was quantified by densitometry normalizing to that of β-actin, with the control level arbitrarily set to 1. D, curcumin inhibited Aurora A kinase activity. After treatment, total protein was subjected to Western blot analysis. Anti-phospho-Aurora A (Thr288) and anti-phospho-histone H3 (Ser10) antibodies served as probes. **, $p < 0.01$. Results are representative of three independent experiments.

Aurora A promoter activity in a dose-dependent manner. In summary, curcumin suppresses Aurora A at the transcriptional level through down-regulation of the promoter activity.

Curcumin Significantly Inhibited Aurora A Protein Expression and Thus Kinase Activity. To determine the protein level of Aurora A in response to curcumin, T24 cells were incubated with 0 to 30 μM curcumin for 12 to 48 h. A significant decrease ($p < 0.01$) in the level of Aurora A protein was observed when T24 cells were treated with 30 μM curcumin for 48 h by Western blot analysis (Fig. 1C). Because phosphorylation is required for Aurora A kinase activity (Andrésson and Ruderman, 1998) and Aurora A is autophosphorylated in its activation loop on Thr288 (Ohashi et al., 2006), the effect of curcumin on the baseline phosphorylation level of phospho-Thr288-Aurora A was examined. As shown in Fig. 1D and Supplemental Fig. 1, a significant inhibition ($p < 0.01$) of phospho-Thr288-Aurora A expression was observed when the cells were treated with 30 μM curcumin for 48 h. Histone H3 is a direct downstream substrate of Aurora A (Hirota et al., 2005). To confirm the suppression in Aurora A kinase activity by curcumin, the phosphorylation level of histone H3 on Ser10 was determined. Consistent with the results of phospho-Thr288-Aurora A expression, a significant suppression ($p < 0.01$) of phospho-histone H3 was detected when the cells were treated with 30 μM curcumin for 48 h (Fig. 1D; Supplemental Fig. 1). Collectively, curcumin effectively suppressed Aurora A protein expression and subsequently caused the decrease of Aurora A kinase activity at the concentration of 30 μM curcumin for 48 h.

Curcumin Preferentially Suppressed the Growth of Cells with a Higher Expression Level of Aurora A. To test the hypothesis that the anticancer activity of curcumin is Aurora A-related, grade III human bladder cancer T24 cells and immortalized uroepithelial E7 cells with different Aurora A expression levels (Tseng et al., 2006) were used. Because the Aurora A protein expression level varies during cell cycle progression (Honda et al., 2000), T24 and E7 cells were synchronized at G_2/M before experimental treatment. As shown in Fig. 2A, T24 cells expressed a higher level of Aurora A compared with E7 cells. It is noteworthy that T24 cells also exhibited a higher proliferation rate (Fig. 2B) and invasion ability (Fig. 2C). To evaluate the association of Aurora A expression with curcumin-related growth inhibition, the effect of curcumin on the growth of these two cell lines were examined by MTT assay. As shown in Fig. 2D, E7 cells are more resistant to curcumin treatment, and curcumin suppressed the growth of T24 cells in a dose- and time-related manner. The 50% inhibitory concentrations (IC_{50}) of T24 cells are 47.1 ± 6.5, 32.2 ± 3.3, and 30.7 ± 5.6 μM at 24, 48, and 72 h (Fig. 2D). More than 60% of E7 cells still proliferate in the presence of 50 μM curcumin for 72 h. The curcumin treatment concentration of 30 μM was used in the following experiments. These results suggest that curcumin preferentially inhibits the growth of T24 cells with higher expression levels of Aurora A.

Effect of Curcumin on Mitotic Events of T24 Cells by Immunofluorescent and Confocal Microscopy. Aurora A protein expression and distribution in T24 cells were investigated under confocal microscope. The results showed that Aurora A is located at centrosomes and spindles of T24 cells without treatment (Fig. 3). Consistent with the data obtained from the Western blot analysis (Fig. 1C), a decrease in the intensity of Aurora A staining was detected and reached the lowest level at 48 h after treatment with 30 μM curcumin (Fig. 3). Although Aurora A is an oncoprotein, it is also required for mitotic entry in human cells (Prigent and Giet, 2003). To assess the effect of curcumin-induced suppression of Aurora A on mitosis, the percentage of mitotic cells was counted by immunofluorescent microscopic analysis of spindle formation (Marumoto et al., 2005) and centrosome

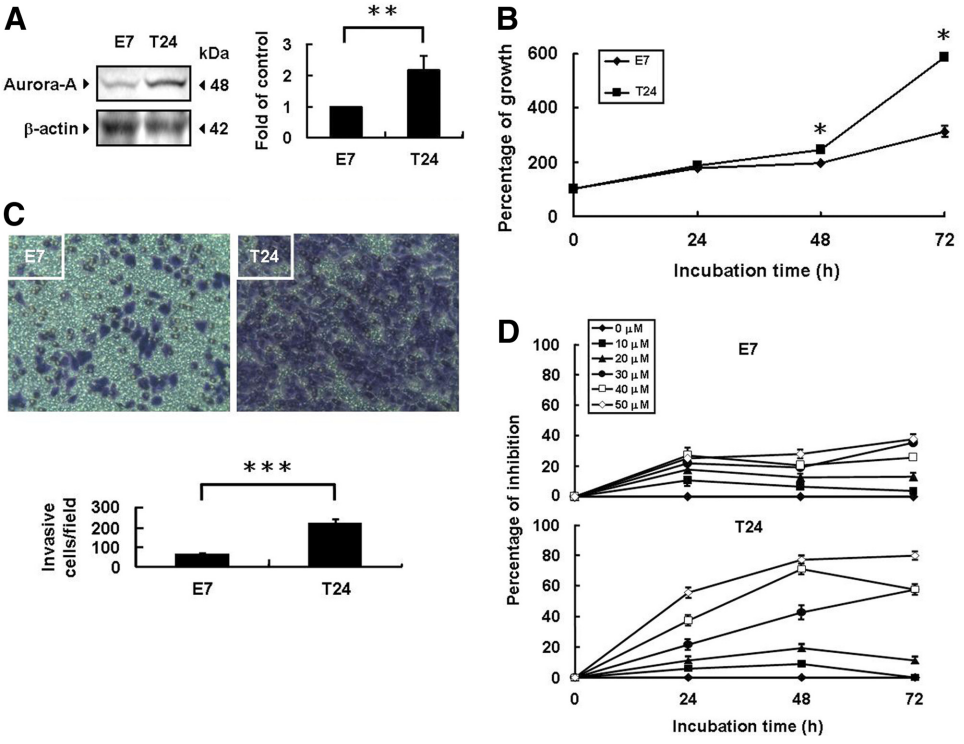

Fig. 2. Characteristic of cells with different expression levels of Aurora A and effect of curcumin on growth of these cells. A, Aurora A protein expression in E7 and T24 cells. Cells were treated with nocodazole (400 nM) for 36 h. After release from nocodazole-induced G_2/M block, total protein was subjected to Western blot analysis. **, $p < 0.01$. B, growth rate of E7 and T24 cells in the absence of curcumin. Values are means ± S.E.M. and are presented as a percentage of growth with the control level arbitrarily set to 100. *, $p < 0.05$. C, invasion ability of E7 and T24 cells in the absence of curcumin. The Matrigel invasion assay was performed in triplicate. The invasive cells in five random fields were counted for each assay. Data are means ± S.E.M. of invasive cells. ***, $p < 0.001$. D, growth inhibition of E7 and T24 cells in response to curcumin. Values are means ± S.E.M. and are presented as percentage of inhibition with the control level arbitrarily set to 0. Results are representative of three independent experiments.

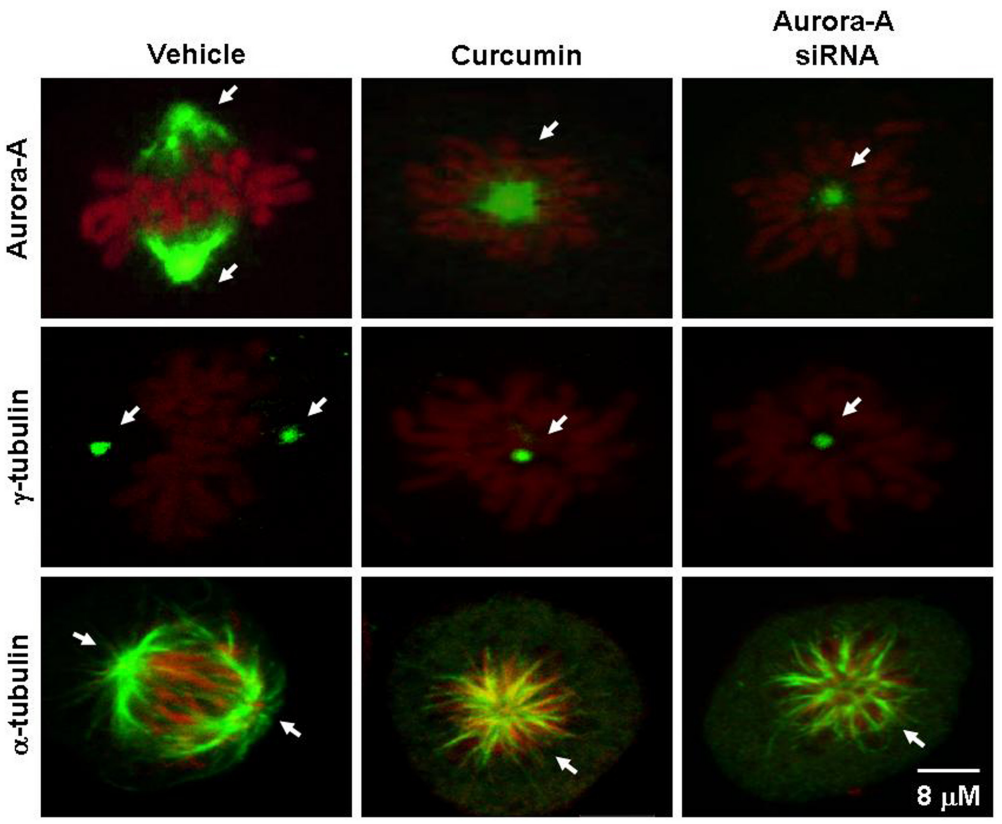

Fig. 3. Morphologic analysis of T24 cells in response to curcumin or Aurora A siRNA. T24 cells were treated with or without curcumin (30 μM) for 48 h or transfected with Aurora A siRNA for 4 h. After removal of the transfection medium, the cells were incubated with fresh complete DMEM for another 44 h. The cells were then stained with anti-Aurora A (green), anti-γ-tubulin (green), or anti-α-tubulin (green) antibody. DNA morphologies were visualized by incubating the cells with PI (red, 5 μg/ml). The images were taken by confocal microscopy. White arrows indicate the centrosomes. Results are representative of three independent experiments.

separation (Nigg, 2001). Morphology of mitotic spindles was also examined. The results revealed that the percentage of cells undergoing cell division was decreased by curcumin in a dose-related manner, ranging from 10.9% in untreated cells to 9.2, 6.5, and 3.6% in cells treated with 10, 20, and 30 μM curcumin (Table 1). The degree of Aurora A siRNA-induced decrease in cell division (2.2%) was similar to that of 30 μM curcumin. Among these mitotic cells, the curcumin-induced monopolar phenotype was increased in a dose-related manner, ranging from 2.9% in untreated cells to 10.6, 23.5, and 60.5% in cells treated with 10, 20, and 30 μM curcumin. It is noteworthy that 30 μM curcumin acted similarly to Aurora A siRNA (65%), resulting in a similar percentage of cells with a monopolar phenotype (Table 1; Fig. 3). Induction of a monopolar phenotype was also reported in curcumin-treated MCF-7 cells (Banerjee et al., 2010). To confirm the effect of curcumin on assemblies of bipolar spindles, mitotic centrosomes in T24 cells were examined by confocal microscopy using antibody against γ-tubulin, a component of pericentriolar material (Marumoto et al., 2003). As shown in Table 1 and Fig. 3, the percentage of cells exhibiting monopolar spindle was elevated in a dose-related manner, ranging from 0.0% in untreated cells to 5.2, 17.8, and 52.7% in cells treated with 10, 20, and 30 μM curcumin. Aurora A siRNA (58.1%) acted similarly to 30 μM curcumin. These results were consistent with those seen by use of anti-Aurora A antibody (Table 1). Mitotic spindle and chromosome alignment were also examined in dividing T24 cells stained with anti-α-tubulin antibody, because formation of abnormal mitotic spindles is a phenotype of Aurora A inhibition (Marumoto et al., 2003). In the untreated cells, normal bipolar mitotic spindles with properly aligned chromosomes were displayed (Fig. 3). However, curcumin-treated (20 μM or more) or Aurora A siRNA-transfected cells exhibited misaligned spindle with various chromosome alignment defects (Fig. 3; Supplemental Fig. 2).

In Aurora A-stained mitotic cells, curcumin was capable of inducing multiple centrosomes, especially when T24 cells were treated with 20 μM curcumin for 48 h (36.0%) (Table 1; Supplemental Fig. 2). A small percentage of cells displaying multiple centrosomes was observed in untreated (3.0%), 10 μM curcumin-treated (6.8%), and 30 μM curcumin-treated (5.7%) groups. Multiple centrosomes were scarcely found in the cells treated with Aurora A siRNA. Instead, most of these cells (65.0%) exhibited a monopolar structure. In the anti-γ-tubulin-stained mitotic cells, the percentage of cells exhibiting multiple centrosomes was 3.0, 5.2, 31.0, 2.8, and 0% for the cells treated with 0, 10, 20, and 30 μM curcumin and Aurora A siRNA, respectively (Table 1; Supplemental Fig. 2). These observations on multiple centrosomes were consistent

TABLE 1
Effect of curcumin on cell division and morphology of mitotic spindles
T24 cells were treated with curcumin for 48 h or transfected with Aurora-A siRNA. The percentage of mitotic cells and mitotic phenotype was scored based on Aurora-A or γ-tubulin fluorescence in addition to DNA morphology by immunofluorescent microscopic analysis (the number of cells for each condition was larger than 300), using Aurora-A siRNA as a positive control. Data were a quantification of Fig. 3 and Supplemental Fig. 2. Results are representative of three independent experiments.

	Curcumin				Aurora-A siRNA
	0 μM	10 μM	20 μM	30 μM	
			%		
Anti-Aurora-A dividing cells	10.9	9.2	6.5	3.6	2.2
Multiple centrosomes	3.0	6.8	36.0	5.7	0.0
Monopolar spindle	2.9	10.6	23.5	60.5	65.0
Anti-γ-tubulin					
Multiple centrosomes	3.0	5.2	31.0	2.8	0.0
Monopolar spindle	0.0	5.2	17.8	52.7	58.1

with those examined by using anti-Aurora A antibody. In agreement with our previously observation using anti-Aurora A and anti-γ-tubulin antibodies (Table 1), in anti-α-tubulin-stained mitotic cells, a significant multiple polar phenomenon was displayed in the cells treated with 20 μM curcumin (Supplemental Fig. 2).

Curcumin Treatment Interfered with Mitotic Entry and Exit of T24 Cells. A decrease in Aurora A protein expression by Aurora A siRNA results in G_2/M cell cycle arrest (Du and Hannon, 2004). To examine the effect of curcumin on cell cycle progression, asynchronized T24 cells were treated with curcumin (0–30 μM) for 48 h and then stained with PI before flow cytometric analysis. Compared with the untreated cells (11.3 ± 3.6%), a significant increase ($p < 0.01$) in the G_2/M phase was observed in the cells treated with 30 μM curcumin (23.9 ± 0.6%), similar to the degree of that in the cells transfected with Aurora A siRNA (21.3 ± 0.6%) (Fig. 4A; Supplemental Table 1). To directly analyze the effect of curcumin on mitotic progress, T24 cell cycle was synchronized at G_0/G_1. Flow cytometric analysis revealed that 57.0 ± 2.6% of the cells remained at the G_0/G_1 phase at time 0. After release from the starvation block, the cells were treated with or without curcumin for 6 to 12 h. As shown in Fig. 4B and Supplemental Table 2, the cells without curcumin rapidly completed the S phase (entered at 6 h and exited at 12 h), and no significant accumulation in G_2/M was displayed at 12 h. However, the cells cultured with curcumin showed a delay in mitotic entry and exit characterized by significant increases ($p < 0.01$) in the S and G_2/M phases (46.7 ± 1.1 and 29.8 ± 1.1%) compared with the untreated cells (31.9 ± 1.9 and 14.6 ± 6.0%) at 12 h. Similar results in HeLa cells have been reported by Tyler et al. (2007). Incon-

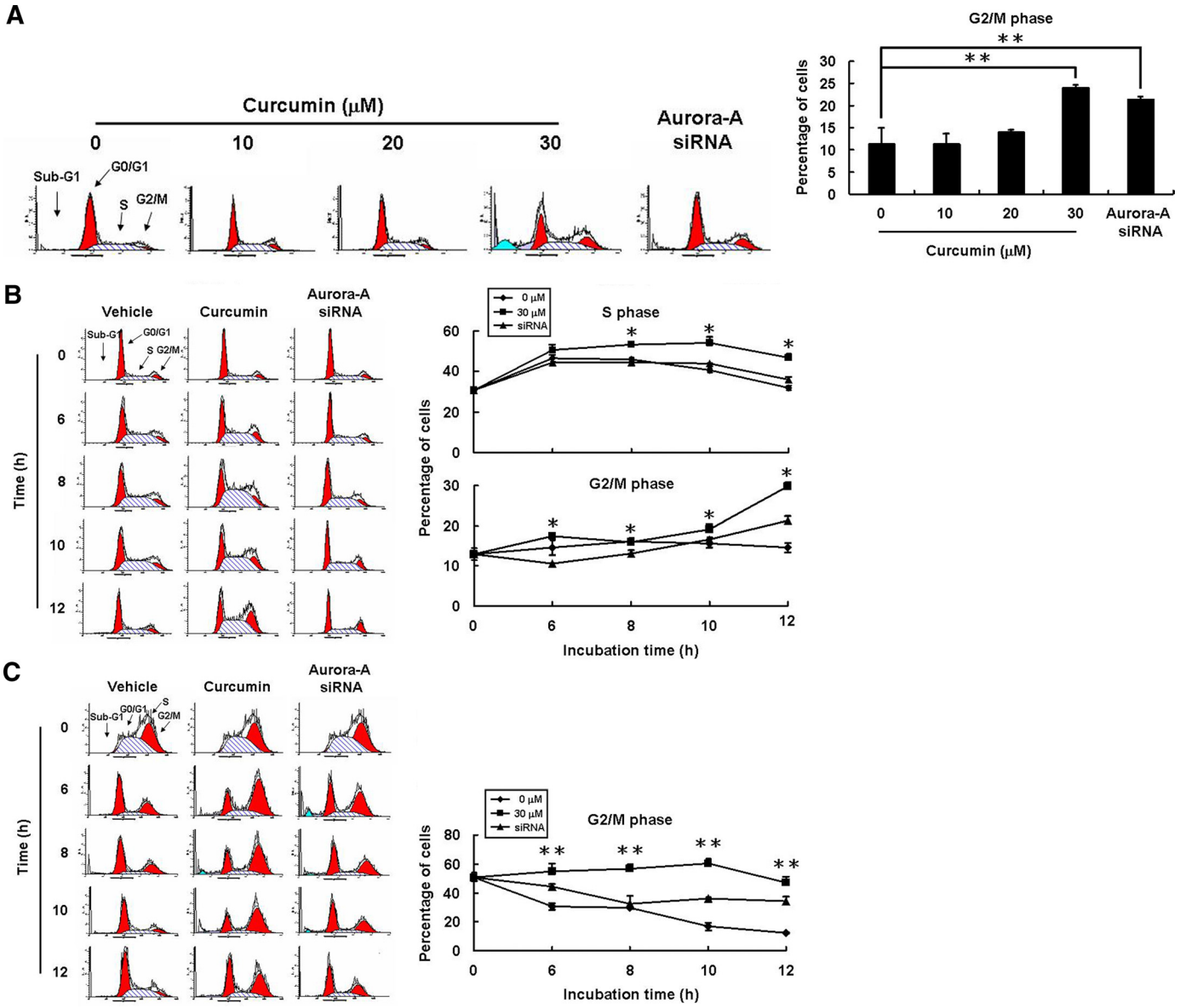

Fig. 4. Inhibition of cell cycle progression by curcumin and Aurora A siRNA. A, effect of curcumin on cell cycle distribution of asynchronized T24 cells. Cells were treated with curcumin for 48 h or transfected with Aurora A siRNA. All of the cells were stained with PI (40 μg/ml) before flow cytometric analysis. Data are presented as means ± S.E.M. **, $p < 0.01$. B, effect of curcumin on cell cycle distribution of T24 cells after starvation (cultured with DMEM containing 1% FBS for 24 h). *, $p < 0.05$. C, effect of curcumin on cell cycle distribution of T24 cells synchronized by nocodazole (400 nM) for 24 h. **, $p < 0.01$. Results are representative of three independent experiments.

sistent with the results of the cells treated with curcumin, significant elevations in the S and G_2/M phases (36.1 ± 1.5 and 21.3 ± 1.1%) were also observed when the cells were treated with Aurora A siRNA at 12 h. Throughout the experiments, Aurora A protein expressions were inhibited in the cells treated with either curcumin or Aurora A siRNA (Supplemental Fig. 3).

To confirm the inhibition of Aurora A on the G_2/M population, T24 cells were synchronized at mitosis by the blocker nocodazole (Szüts and Krude, 2004) before flow cytometric analysis. When the cell cycle was released from the G_2/M block (50.7 ± 1.4%), the cells were treated with or without curcumin for 6 to 12 h. Figure 4C and Supplemental Table 3 show that the untreated cells immediately exited the G_2/M phase (30.6 ± 1.3% at 6 h, 16.8 ± 1.4% at 10 h, and 12.3 ± 0.2% at 12 h) and entered the G_0/G_1 and then the S phase. In contrast, curcumin still blocked the cells at the G_2/M phase (47.3 ± 2.3%) 12 h after release from the G_2/M block. Aurora A siRNA also delayed cell cycle progression but to a less extend (44.1 ± 1.0% at 6 h and 34.2 ± 1.8% at 12 h). Throughout the experiment, Aurora A protein expression (Supplemental Fig. 4) and kinase activity (Supplemental Fig. 5) were suppressed by the treatment with curcumin.

Effect of Ectopic Aurora A on Curcumin-Treated T24 Cells. To verify the role of Aurora A in curcumin-induced mitotic events and cell cycle progression, T24 cells ectopically expressing wild-type Aurora A were treated with 30 μM curcumin for 48 h. As shown in Fig. 5A, ectopic Aurora A increased the total amount of Aurora A protein expression. Curcumin down-regulated the endogenous Aurora A but not the ectopic Aurora A expression. Immunofluorescent microscopic analysis of spindle formation and centrosome separation revealed that ectopic Aurora A restored the curcumin-induced decrease in the percentage of dividing cells from 3.6 to 4.0% (Table 2). Among these dividing cells, ectopic Aurora A decreased the percentage of curcumin-induced increase in monopolar spindles from 50.0 to 18.7%. Flow cytometric analysis further indicated that ectopic Aurora A significantly decreased curcumin-induced accumulation of the cells at the G_2/M phase (Fig. 5B; Supplemental Table 4).

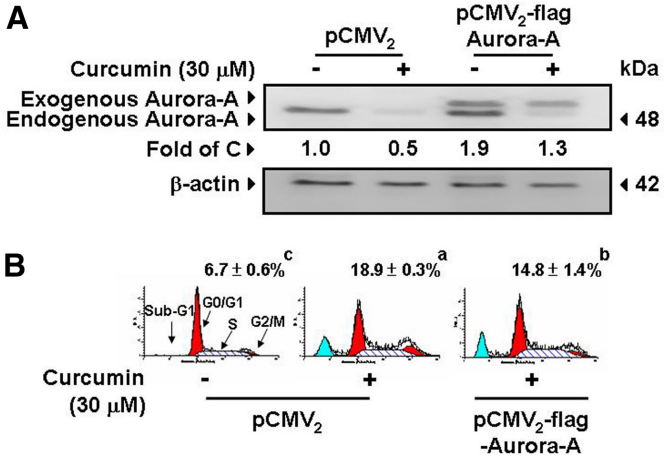

Fig. 5. Ectopic Aurora A restored the effect of curcumin. A, protein expression of endogenous and ectopic Aurora A. After treatment, total protein was subjected to Western blot analysis. Anti-Aurora A antibody was served as a probe. B, ectopic Aurora A on cell cycle progression. After treatment, cells were stained with PI (40 μg/ml) before flow cytometry. The percentages in the figure indicate the proportion of cells in G_2/M phase. Data are presented as means ± S.E.M. Means without a common letter differ, $p < 0.05$. Results are representative of three independent experiments.

TABLE 2

Effect of ectopic Aurora-A on cell division and mitotic events of curcumin-treated T24 cells

T24 cells transfected with wild-type Aurora-A (pCMV2-flag-Aurora-A) were treated with curcumin for 48 h. The percentages of mitotic cells and mitotic phenotype were scored on the basis of Aurora-A fluorescence and DNA morphology by immunofluorescent microscopy analysis (the number of cells for each condition was larger than 300). Results are representative of three independent experiments.

	Control	30 μM Curcumin	pCMV2-flag-Aurora-A + 30 μM Curcumin
		%	
Anti-Aurora-A dividing cells	7.2	3.6	4.0
Multiple centrosomes	8.0	8.3	12.5
Monopolar spindle	4.0	50.0	18.7

Discussion

In the present study, we found that curcumin, a natural compound, significantly inhibited Aurora A protein expression and thus kinase activity, associating with destruction of normal bipolar spindles and delay of mitotic progression at the G_2/M phase. Administration of Aurora A siRNA and ectopic expression of Aurora A further demonstrate that the antitumor mechanism of curcumin is Aurora A-related.

Of importance, curcumin-induced cell growth inhibition was preferentially higher in T24 cells (Fig. 2D) with a higher expression level of Aurora A (Fig. 2A), suggesting a potential pharmacological window for a curcumin therapeutic response in Aurora A-high cancer cells. T24 is an Aurora A-overexpressing grade III human bladder cancer cell line (Lin et al., 2006). Although there are disputes about whether Aurora A overexpression is correlated with higher-grade tumors and poor prognosis (Marumoto et al., 2005), suppression of Aurora A has become a target for cancer therapy. Different Aurora A kinase inhibitors with different specificities are in different phases of clinical trial (Karthigeyan et al., 2010).

Aurora A activity can be regulated by transcription of the Aurora A gene and/or phosphorylation of Aurora A protein (Karthigeyan et al., 2010). In this study, down-regulation of Aurora A by curcumin seems to be at the transcription level because inhibition of phospho-Aurora A (Thr288) and phospho-histone H3 (Ser10) (Fig. 1D) was paralleled by significant decreases in Aurora A mRNA expression (Fig. 1A). Curcumin-induced inhibition in Aurora A expression may not be tissue-specific. Our unpublished data indicate that mRNA expression in human hepatocellular carcinoma Hep 3B cells and protein level and kinase activity in human breast cancer MCF-7 cells were also significantly suppressed in response to curcumin. The reason for the inconsistencies in the pattern of curcumin-induced decreases in mRNA (Fig. 1A) and protein (Fig. 1C) expressions and luciferase activity (Fig. 1B) may be that the luciferase activity determined the immediate response of Aurora A promoter activity to curcumin treatment, whereas mRNA and protein expressions represented total accumulation of post-transcriptional and post-translational events on Aurora A mRNA and protein, respectively, in the cells. Curcumin is a compound readily available in food worldwide, and it has been demonstrated to be safe in humans (Goel et al., 2008). Recent reports further indicate that

inhibition of Aurora A enhanced tumor radiosensitivity (Tao et al., 2007) and chemotherapeutic efficacy (Briassouli et al., 2007), implying the importance of Aurora A kinase targeting in combination with standard clinical treatments.

Inhibition of Aurora A activity in tumor cells leads to impaired chromosome alignment and subsequent cell death (Carvajal et al., 2006). Repression of Aurora A also effectively blocks cell growth and thus induces apoptosis in cancer cells (Huang et al., 2008). In the present study, the curcumin-induced decrease in the percentage of dividing cells was consistent with use of Aurora A siRNA (Table 1). Furthermore, the curcumin-induced inhibition in cell division was attenuated by ectopic expression of Aurora A (Table 2). Similar effects were observed on cell cycle arrest at G_2/M (Figs. 4, A–C and 5B; Supplemental Tables 1–4) and abnormal centrosomal morphology and spindle formation (Fig. 3; Tables 1 and 2; Supplemental Fig. 2). It is noteworthy that these curcumin-induced defects in mitotic events were paralleled by a significant increase in tumor cell G_2/M arrest, suggesting that curcumin-induced suppression in cell growth (Fig. 2D) was associated in part with inhibition of Aurora A activity. Of interest, we observed the induction of apoptosis, characterized by the accumulation of cells at the sub-G_1 phase (Figs. 4A and 5B; Supplemental Tables 1 and 4) and increase in cells with externalized phosphatidylserine (Supplemental Fig. 6), suggesting that the inhibitory role of curcumin was due to the induction of cell arrest and apoptosis.

Curcumin itself is a potent anticancer agent. Phase III clinical trials are undergoing to evaluate the effects of curcumin against pancreatic cancer and colon cancer (Hatcher et al., 2008). Possible antitumor activity of curcumin includes induction of tumor apoptosis and inhibition of tumor proliferation, invasion, angiogenesis, and metastasis (Hatcher et al., 2008). Numerous targets regulated by curcumin have been reported, consisting of kinases, enzymes, growth factors, cytokines, and transcription factors (Anand et al., 2008). Among them, as many as 33 different proteins can physically bind to curcumin (Fu and Kurzrock, 2010). Partial recovery of the curcumin-induced phenomena by the administration of ectopic Aurora A (Fig. 5B; Table 2; Supplemental Table 4) indicates that there are Aurora A-unrelated pathways involved in curcumin-induced anticancer mechanism.

In several studies, curcumin-induced p53-dependent apoptosis and G_2/M arrest have been reported (Choudhuri et al., 2005; Liu et al., 2007). p53, a tumor suppressor and a key regulator of cell survival and cell cycle progression, is a physiological substrate of Aurora A (Karthigeyan et al., 2010). Aurora A phosphorylates p53 at Ser315 and Ser215, by which the DNA binding activity and transactivation activity of p53 are suppressed (Liu et al., 2004). Because T24 is a p53 mutated cell line (Hinata et al., 2003), the antitumor efficacy of curcumin proceeding via inhibition of Aurora A in T24 cells must be p53-independent. The lack of p53 function in cells may increase resistance to ionizing radiation due to loss of growth arrest and/or apoptosis mechanisms (Hinata et al., 2003); however, curcumin-induced inhibition of Aurora A can sensitize the cells without functional p53 (Tao et al., 2007).

In conclusion, the characteristics of Aurora A inhibition by curcumin were consistent with those using Aurora A siRNA, which was restored by ectopic expression of Aurora A. Suppression of Aurora A has been a promising strategy for cancer therapy, and the antitumor efficacy of curcumin has been proven in clinical trials. The discovery of curcumin-induced inhibition of oncogene Aurora A in conjunction with its safety and ready availability in food worldwide of curcumin suggests its chemoprevention and chemotherapeutic potential in human cancers.

Authorship Contributions

Participated in research design: Liu, Cheng, Huang, and Su.
Conducted experiments: Ke.
Wrote or contributed to the writing of the manuscript: Su.

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