Clinical Cancer Research

# Aurora Kinase A Promotes Ovarian Tumorigenesis through Dysregulation of the Cell Cycle and Suppression of BRCA2

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## Abstract

**Purpose:** Aurora kinase A (Aurora-A) is known to regulate genomic instability and tumorigenesis in multiple human cancers. The underlying mechanism, however, is not fully understood. We examined the molecular mechanism of Aurora-A regulation in human ovarian cancer.

**Experimental Design:** Retrovirus-mediated small hairpin RNA (shRNA) was used to silence the expression of Aurora-A in the ovarian cancer cell lines SKOV3, OVCA432, and OVCA433. Immunofluorescence, Western blotting, flow cytometry, cytogenetic analysis, and animal assay were used to test centrosome amplification, cell cycle alteration, apoptosis, DNA damage response, tumor growth, and genomic instability. Immunostaining of BRCA2 and Aurora-A was done in ovarian, pancreatic, breast, and colon cancer samples.

**Results:** Knockdown of Aurora-A reduced centrosome amplification, malformation of mitotic spindles, and chromosome aberration, leading to decreased tumor growth. Silencing Aurora-A attenuated cell cycle progression and enhanced apoptosis and DNA damage response by restoring p21, pRb, and BRCA2 expression. Aurora-A was inversely correlated with BRCA2 in high-grade ovarian serous carcinoma, breast cancer, and pancreatic cancer. In high-grade ovarian serous carcinoma, positive expression of BRCA2 predicted increased overall and disease-free survival, whereas positive expression of Aurora-A predicted poor overall and disease-free survival ($P < 0.05$). Moreover, an increased Aurora-A to BRCA2 expression ratio predicted poor overall survival ($P = 0.047$) compared with a decreased Aurora-A to BRCA2 expression ratio.

**Conclusion:** Aurora-A regulates genomic instability and tumorigenesis through cell cycle dysregulation and BRCA2 suppression. The negative correlation between Aurora-A and BRCA2 exists in multiple cancers, whereas the expression ratio of Aurora-A to BRCA2 predicts ovarian cancer patient outcome. *Clin Cancer Res; 16(12); 3171–81. ©2010 AACR.*

Genomic instability plays a crucial role in the onset and progression of human tumors. The serine/threonine kinase Aurora-A (*AURKA*) acts to maintain cell division through regulation of centrosome separation, bipolar spindle assembly, and chromosome segregation (1, 2). However, Aurora-A is commonly amplified to induce genomic instability in many human cancers, including breast (3), pancreatic (4), bladder (5), gastric (6), and colorectal (7) cancers. Overexpression of Aurora-A can transform mouse NIH/3T3 cells by inducing centrosome amplification and aneuploidy (2). Aurora-A also interacts with p53 and BRCA1 to regulate the cell cycle checkpoint and to maintain genomic integrity by phosphorylating p53 at Ser 215 and Ser 315 (8, 9) or BRCA1 at Ser 308 (10).

In ovarian cancer, activation or overexpression of Aurora-A is found in both cancer cell lines (11, 12) and tumor specimens (13, 14) and is associated with a poor prognosis in patients with cancer (14, 15). Aurora-A may promote tumorigenesis through interaction with the cell cycle regulatory protein E2F3 (16), NF-κB (17), Akt (18), c-Myc, and p53 (19). However, detailed mechanisms of how Aurora-A signaling induces genomic instability and other downstream targets are unknown. In this study, we used ovarian cancer cell lines to investigate the role of Aurora-A and its associated mechanisms in genomic instability and tumorigenesis.

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## Translational Relevance

In this study, we show that Aurora kinase A (Aurora-A) promotes ovarian tumorigenesis through dysregulation of the cell cycle and suppression of BRCA2. We found a negative correlation between Aurora-A and BRCA2 in multiple cancer types, including ovarian, pancreatic, and breast cancers, which may represent a general mechanism associated with Aurora-A–mediated cancer progression. Moreover, the expression ratio of Aurora-A and BRCA2 can be used to predict ovarian cancer outcomes: increased expression of Aurora-A to BRCA2 predicts poor overall survival whereas decreased level of Aurora-A to BRCA2 indicates favorable overall survival. Thus, our study provides strong clinical evidence that the ratio of Aurora-A to BRCA2 expression can be used as a marker to predict the prognosis in human ovarian cancer.

## Materials and Methods

### Cell lines, small hairpin RNA, retroviruses, and tumor formation

Ovarian cancer cell lines SKOV3, OVCA432, and OVCA433 were obtained from the American Type Culture Collection (ATCC) and were maintained in Eagle's MEM (Lonza Walkersville, Inc.) containing 10% fetal bovine serum, 2 mmol/L L-glutamine, penicillin (100 units/mL), and streptomycin (100 μg/mL). A retroviral vector pBabe/U6/puromycin with Aurora-A small hairpin RNA (shRNA; targeting 5′-GUCUUGUGUCCUUCAAAUU-3′ of Aurora-A mRNA) was constructed to deliver Aurora-A shRNA (labeled as Aurora-Ai) into ovarian cancer cells SKOV3, OVCA432, and OVCA433 using a previously described method (20, 21). The control vector was similarly constructed by directly inserting oligonucleotides encoding small interfering RNA against mRNA encoding green fluorescence protein (GFPi) into pBabe/U6/puromycin (22). The retrovirus infection was done according to our previously published method (21). The resulting cell lines after infection and selection were named SKOV3/GFPi, SKOV3/Aurora-Ai, OVCA432/GFPi, OVCA432/Aurora-Ai, OVCA433/GFPi, and OVCA433/Aurora-Ai, respectively.

For *in vivo* tumor growth, $5 \times 10^5$ cells for SKOV3/GFPi and SKOV3/Aurora-Ai or $6 \times 10^6$ cells for OVCA432/GFPi, OVCA432/Aurora-Ai, OVCA433/GFPi, and OVCA433/Aurora-Ai were s.c. injected into 4- to 6-week-old BALB/c *nu/nu* mice (NCI Frederick Cancer Research Facility) according to the protocol approved by the institutional committee for animal experiments (20). Each cell line was bilaterally injected into 6 mice, for a total of 12 injections. Tumor burden was recorded as described previously (20).

### Western blotting

Western blotting was done as described previously (21, 23). The primary antibody used to detect Aurora-A was purchased from GeneTex (GTX13824), and the antibody to detect BRCA2 was from R&D Systems (MAB2476). The antibodies to p21 (sc-817), pRb (sc-7950), cyclin D1 (sc-246), Cdk4 (sc-260), Cdk6 (sc-7961), cyclin E (sc-247), Cdk2 (sc-6248), cyclin B1 (sc-752), and Rad51 (sc-8349) were purchased from Santa Cruz Biotechnology. The antibody to β-actin was from Sigma-Aldrich.

### Assays for cell proliferation and anchorage-independent colony formation

To test cell proliferation, $1 \times 10^4$ cells for all cell lines with Aurora-Ai or GFPi were seeded into 12-well plates (each cell line in 10 wells). Cells were incubated at 37°C in an atmosphere of 5% $CO_2$ and 95% air and harvested from 2 wells for each cell line every 2 days and counted individually for a total of 10 days (5 counts). The mean number of cells was recorded. The assay was repeated three times in duplicate.

For anchorage-independent colony formation, ovarian cancer cell lines infected with GFPi (SKOV3/GFPi, OVCA432/GFPi, OVCA433/GFPi) or Aurora-Ai (SKOV3/Aurora-Ai, OVCA432/Aurora-Ai, OVCA433/Aurora-Ai) were used to carry out soft agar assay according to our previous publications (24). Briefly, $5 \times 10^4$ cells were suspended in 2 mL of medium with 0.35% agarose (Life Technologies), and the suspension was placed on top of 5 mL of solidified 0.7% agarose. Triplicate cultures of each cell type were maintained for 14 days at 37°C in a 5% $CO_2$ atmosphere, and fresh medium was fed at 7 days. The number of colonies >50 μm (~100 cells) in diameter in each dish was counted at 14 to 20 days. The assay was repeated three times in duplicate.

### Immunofluorescence

Immunofluorescence staining was done according to a published protocol (20). Primary antibodies against Aurora-A (GTX13824), γ-tubulin (D-10), and BRCA2 (MAB2476) were obtained from GeneTex, Santa Cruz Biotechnology, and R&D Systems, respectively. DNA dye To-Pro-3 was obtained from Molecular Probes. The secondary antibodies used were either FITC-conjugated against mouse IgG or Texas red–conjugated against rabbit IgG (Jackson ImmunoResearch Laboratory). For DNA damage foci examination, cells were first treated with γ-irradiation (10 Gy), then incubated for 3 hours before immunofluorescence staining was done using antibodies against BRCA2 and Rad51. All stained cells were examined and photographed with an Olympus FV500 confocal fluorescence microscope.

### Cytogenetic analysis

SKOV3/GFPi, SKOV3/Aurora-Ai, OVCA432/GFPi, OVCA432/Aurora-Ai, OVCA433/GFPi, and OVCA433/Aurora-Ai cells were fed for 24 hours and collected for chromosome preparation using standard procedures (23). This assay was done by the Molecular Cytogenetics Core Facility in the Department of Genetics at The University of Texas M.D. Anderson Cancer Center. The assay was repeated twice.

**Examination of cell cycle and cell apoptosis**

Cells (1-2 × $10^6$) were harvested, washed twice using 1 × PBS, and resuspended in 200 μL of 1 × PBS. The cells were fixed with 4 mL of cold 75% ethanol at 4°C for a minimum of 4 hours and then washed twice with 1 × PBS. The cells were then resuspended in 500 μL of 1 × PBS and stained with 200 μL of propidium iodide (50 μL/mL; Sigma-Aldrich) and 20 μL of RNase (1 mg/mL; Sigma-Aldrich) in a 37°C water bath for 15 to 20 minutes. Cell cycles were determined by FACStation (BD Biosciences) and analyzed by using CellQuest software and a published method (25). The assay was repeated three times in duplicate.

To detect apoptosis, 1 × $10^5$ cells were stained with Annexin V and propidium iodide according to the Annexin V–fluorescence apoptosis detection kit I (BD Biosciences PharMingen), and subject to analysis with a FACStation equipped with CellQuest software. The percentage of apoptotic cells was calculated in terms of peaks (M2) in the histogram, representing an early apoptotic population (Annexin V+/PI-) among the total cells analyzed (26). The experiment was done in duplicate and repeated three times.

**Tumor samples, tissue microarray construction, and immunohistochemical staining**

The use of tissue blocks and chart review were approved by the Institutional Review Board of the University of Texas M.D. Anderson Cancer Center. Tumor samples and tissue microarray (TMA) construction have been described previously (27). Briefly, ovarian TMA blocks were selected by reviewing H&E-stained sections and were constructed by taking core samples from morphologically representative areas of paraffin-embedded tumor tissues and assembling them on a recipient paraffin block. For each case, two replicate 1-mm core diameter samples were collected, and each was placed on a separate recipient block. All samples were spaced 0.5 mm apart. Five-micrometer sections were obtained from the microarray and stained with H&E to confirm the presence of tumor and to assess the tumor histology. Tumor samples were randomly arranged on the blocks. Sample tracking was based on coordinate positions for each tissue spot in the TMA block; the spots were transferred onto TMA slides for staining. This sample tracking system was linked to a Microsoft Access database containing demographic, clinicopathologic, and survival data for each patient, thereby allowing rapid links between histologic data and clinical features. The array was read according to the given TMA map, each core was scored individually, and the results were presented as the mean of the two replicate cores. Cases without tumor tissue or cores were excluded from the final data analysis. For ovarian cancer, 223 high-grade serous carcinoma cases, including 167 cases before and 56 cases postchemotherapy, were analyzed. Pancreatic cancer arrays (124 cases) have been described previously (28). Breast (208 cases) and colon (210 cases) tissue arrays were obtained from a commercial resource (Pantomics) without identifiers.

TMA slides were treated and stained according to a previously published method (27). Briefly, tissue slides were deparaffinized in xylene and rehydrated in a graded series of ethanol, and sections were subject to antigen retrieval by boiling in 0.01 mol/L sodium citrate buffer (pH 6.0) in a microwave oven for 10 minutes. After blocking endogenous peroxidase activity with 0.3% hydrogen peroxide and blocking nonspecific protein binding with 1.5% normal goat serum, the sections were incubated overnight with primary antibodies at 4°C in a humid chamber. The primary antibody against Aurora-A (GTX13824, monoclonal antibody, Genetax) or BRCA2 (MAB2476, monoclonal antibody, R&D Systems) was applied with the dilution of 1:200 or 1:100 at 4°C in a humid chamber. A biotin-labeled secondary antibody (Universal Goat Link, Biocare Medical) was added for 15 minutes, followed by horseradish peroxidase (Biocare Medical) for 15 minutes. Tissues were then stained for 5 minutes with 3,3′-diaminobenzidine (Biocare Medical). Sections were lightly counterstained with hematoxylin. The primary antibody was replaced with 1 × PBS as a negative control.

Evaluation of staining intensity for immunohistochemical localization of BRCA2 and Aurora-A was independently done by two pathologists (B. C. and J. L.) in a blinded manner. BRCA2 expression was scored for number of cells with nuclear expression, whereas Aurora-A was scored for both cytoplasm and nuclear staining. The pattern we chose in the analysis was also most consistent with our cell biology results: BRCA2 predominantly functions in the nucleus whereas Aurora-A is a kinase, predominantly functioning in the cytoplasm although its nuclear subcellular localization was found in the ovarian cancer cells and patients' tissues. Thus, cores with <5% of cells positive for BRCA2 (nucleus) and Aurora-A (cytoplasmic and nucleus) were considered as negative (given a score of 0); those with 5% to 20% positive cells were scored as 1; those with 20% to 50% positive cells were scored as 2; and those with >50% positive cells were scored as 3. There were very few cases with >50% positive cells in Aurora-A staining, so an independent score of 3 was not recorded for Aurora-A expression in ovarian cancer. For the statistical analyses of ovarian cancer patient survival, the negative expressions for BRCA2 and Aurora-A were designated B0 and A0, whereas the positive expression of BRCA2 was further subdivided into B1, B2, and B3, and the positive expression of Aurora-A was further divided into A1, A2, and A3 (only A1 and A2 in ovarian cancer) in using criteria described above. The expression correlation between BRCA2 and Aurora-A was analyzed from cases with scores for both BRCA2 and Aurora-A staining. We also evaluated other patterns of subcellular distribution of BRCA2 and Aurora-A, but we failed to find any statistical significant scores with any of clinical parameters.

**Statistical analysis**

The overall survival time was computed as the time from the date of first biopsy to the date of death or last follow-up, whichever occurred first. Data from patients who were alive on the last date of follow-up were censored. The disease-free survival time was computed as

the time from the end of the first-line chemotherapy to the time of relapse. Data from patients alive on the last date of follow-up without recurrence were censored. Relationships between expression of BRCA2 and Aurora-A parameters were analyzed by Pearson's correlation coefficient using SPSS16.0 software. The relationships between BRCA2 or Aurora-A and overall or disease-free survival were analyzed by Kaplan-Meier analysis using SPSS16.0 software. Data were statistically analyzed with Statistica (version 6) software (StatSoft). $P < 0.05$ was considered statistically significant.

## Results

### Silence of Aurora-A reduces cell proliferation and *in vitro* and *in vivo* tumorigenesis

To investigate the role of Aurora-A in ovarian cancer development, we first silenced the expression of Aurora-A in three ovarian cancer cell lines (SKOV3, OVCA432, and OVCA433; Fig. 1A) using retroviral vector-based shRNA. Compared with control ovarian cancer cells infected with retrovirus expressing GFPi (Fig. 1A), the expression of Aurora-A was remarkably decreased in ovarian cancer cells treated with Aurora-Ai (Fig. 1A), which resulted in a marked decrease in colony number as shown by the anchorage-independent growth assay (Aurora-Ai; Fig. 1B), cell proliferation (Aurora-Ai; Fig. 1C), and tumor growth in mice (Aurora-Ai; Fig. 1D). These data suggest that overexpression of Aurora-A is critical in ovarian tumorigenesis.

### Knockdown of Aurora-A decreases centrosome amplification and multipolar spindle formation and leads to genomic instability

Aurora-A is known to regulate bipolar spindle formation and chromosome segregation through centrosome

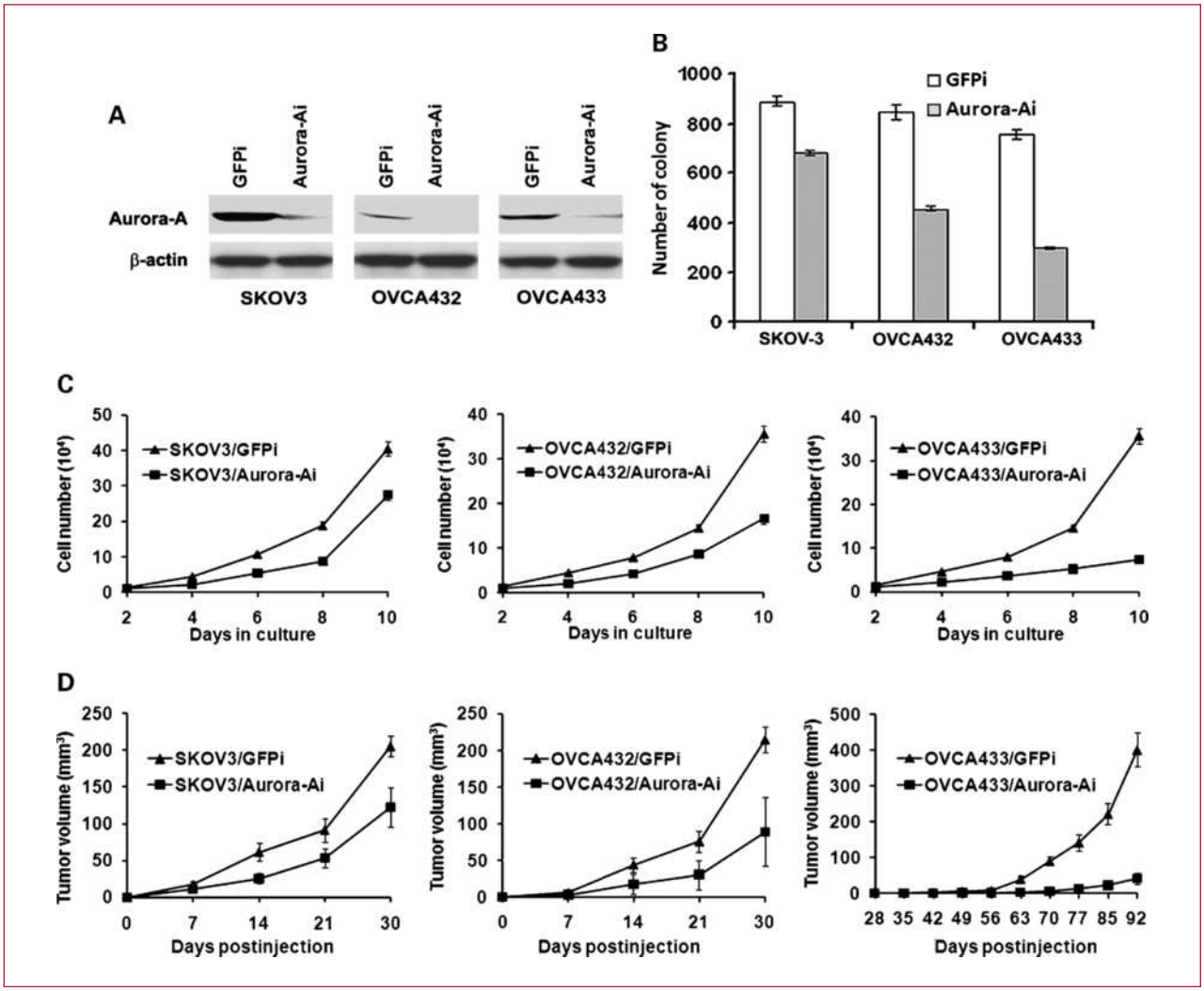


**Fig. 1.** Reduced cell proliferation and tumor formation of ovarian cancer cells after silence of Aurora-A. Analysis of Aurora-A by Western blotting shows that Aurora-A was decreased in ovarian cancer (SKOV3, OVCA432, and OVCA433) cells treated with Aurora-A shRNA (Aurora-Ai) compared with controls treated with GFP shRNA (GFPi, A), which resulted in lower colony numbers (Aurora-Ai, B), reduced cell proliferation (Aurora-Ai, C), and decreased tumor growth (Aurora-Ai, D). Error bars, 95% confidence interval.

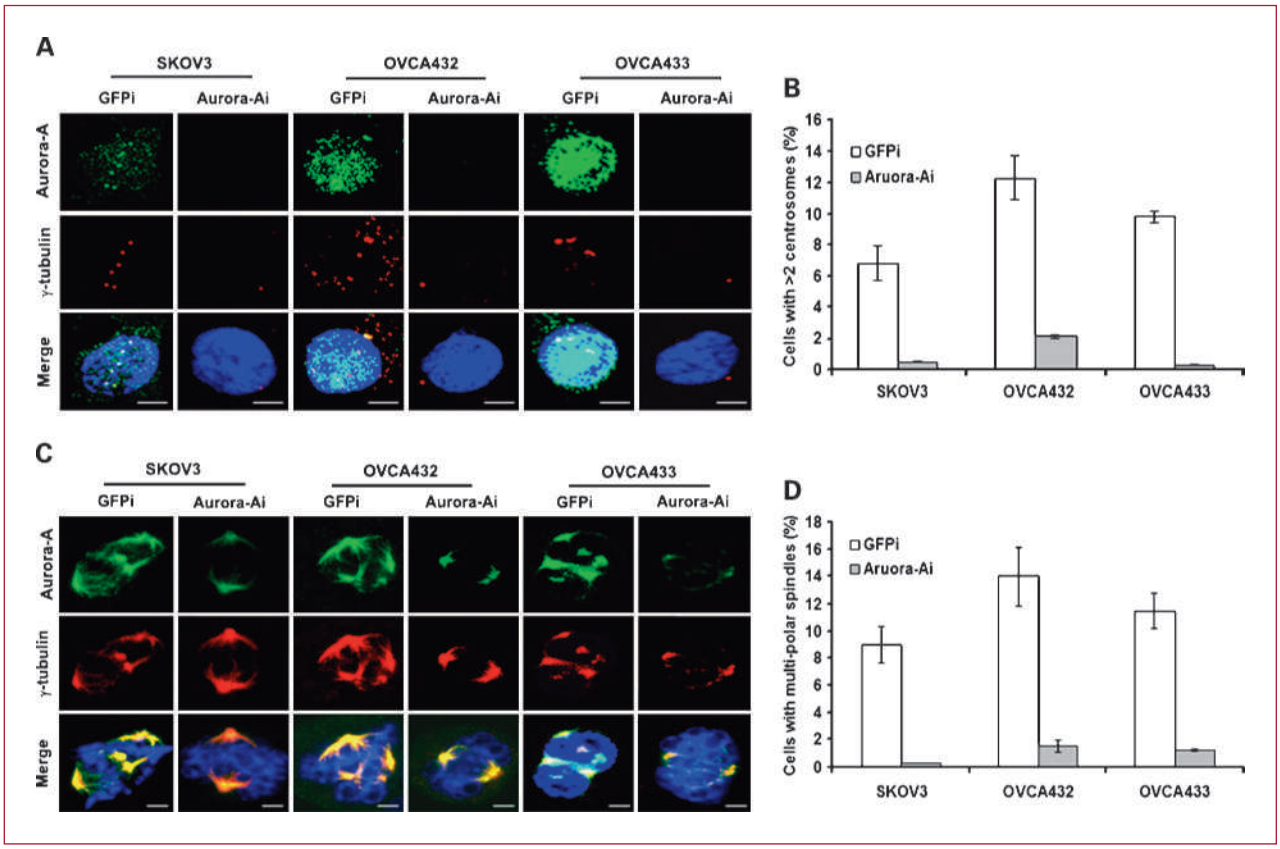


**Fig. 2.** Alteration of centrosome and mitotic spindle formation by knockdown of Aurora-A. A, effect of Aurora-A on centrosome amplification indicated by immunofluorescent staining of Aurora-A and γ-tubulin in quiescent cells. Scale bars, 5 μm. GFPi was used as control. B, quantitative analysis in 300 cells from 12 randomly selected confocal microscopic views indicates the decreased number of cells with >2 centrosomes after cells were treated with Aurora-A shRNA (Aurora-Ai). Error bars, 95% confidence interval of the data collected from three independently repeated experiments. C, centrosome spindle formation in early mitotic cells revealed that numbers of cells with multipolar spindles marked decreased after Aurora-A was silenced (Aurora-Ai). Scale bars, 5 μm. D, quantification of cells with multipolar spindles from 100 cells in 30 randomly selected confocal microscopic views. Error bars, 95% confidence interval of the data collected from three independent experiments.

maturation during cell division (1). We examined the status of centrosomes by immunofluorescent staining for γ-tubulin and Aurora-A in ovarian cancer cells with or without Aurora-Ai. As expected, knockdown of Aurora-A reduced centrosome numbers (Fig. 2A), as indicated by the number of cells with >2 centrosomes in Fig. 2B, showing that Aurora-A controls centrosome amplification. We also observed multipolar mitotic spindles with high frequency in early mitotic cells of SKOV3, OVCA432, and OVCA433 treated with GFPi, whereas knockdown of Aurora-A by shRNA (Aurora-Ai) reduced the formation of multipolar spindles and increased the number of cells with bipolar spindles (Fig. 2C) as shown by a quantitative analysis (Fig. 2D). This result shows that Aurora-A overexpression can disrupt the formation of normal polar mitotic spindles, which may in turn block normal sister chromatid segregation and result in aneuploid daughter cells and centrosome amplification, leading to genomic instability. Taken together, our results suggest that overexpression of Aurora-A promotes centrosome amplification and multipolar spindle formation in ovarian cancer cells.

Because centrosome amplification and multipolar spindle formation affect genomic stability, we found by cytogenetic karyotyping that fewer chromosomal aberrations were observed in cells treated with Aurora-Ai than those treated with GFPi (Table 1). Knockdown of Aurora-A also reduced the number of colchicine-induced anaphase (c-anaphase) cells. The most notable changes in c-anaphase occurred among SKOV3 (>20-fold) and OVCA432 (>10-fold) cells. Consistent with our analysis of multipolar spindles, the proportion of polyploid cells was markedly lower in cells treated with Aurora-A shRNA than in control cells, whereas the population of diploid cells was increased after Aurora-A was silenced (Table 1). Representative karyotypes are shown in Supplementary Fig. S1. Taken together, these results show that Aurora-A plays a crucial role in regulating genomic instability.

### Silence of Aurora-A reduces cell cycle progression through attenuated $G_1$-S transition

Previous studies have shown that Aurora-A may regulate cell cycle progression during the $G_2$-M transition (10, 16).

**Table 1.** Cytogenetic analysis of chromosome abnormalities in ovarian cancer cells after Aurora-A knockdown

| ID | Cell line* | Cells with chromosome aberrations (%) | Cells with DNA breaks (%) | Diploid cells (%) | Polyploid cells (%) | Cells in c-anaphase (%) |
|---|---|---|---|---|---|---|
| 1681 | SKOV3/GFPi | 48.4† | 3 | 36.4† | 58.2† | 45.4† |
| 1682 | SKOV3/Aurora-Ai | 2.9†(↓) | 2.9 | 67.6†(↑) | 29.4†(↓) | 2.9†(↓) |
| 1676 | OVCA432/GFPi | 47.2† | 33.3† | 41.7‡ | 48.3† | 11.1† |
| 1677 | OVCA432/Aurora-Ai | 9.4†(↓) | 3.1†(↓) | 68.7‡(↑) | 21.9†(↓) | 0†(↓) |
| 1678 | OVCA433/GFPi | 23.3† | 16.7† | 66.7‡ | 16.7† | 3.3 |
| 1679 | OVCA433/Aurora-Ai | 11.4†(↓) | 8.6†(↓) | 88.6‡(↑) | 0†(↓) | 0 |

*For each cell line, 30 to 36 cells in metaphase were examined. An increase or a decrease in chromosomal aberrance in terms of DNA breaks, diploidy, polyploidy, and c-anaphase is indicated as ↑ or ↓, respectively.
†$P < 0.01$.
‡$P < 0.05$.

We found that the cell population was increased (~15%) in the $G_0$-$G_1$ phase but decreased (~6%) in the S phase in all three cell lines treated with Aurora-A shRNA, whereas the cell population in the $G_2$-M phase was slightly decreased only in SKOV3 and OVCA433 cells treated with Aurora-A shRNA (Fig. 3A). To explore the potential mechanism, we analyzed major proteins associated with cell cycle regulation by Western blotting. The results in Fig. 3B show that p21 (Cip1/Waf1) and pRb, two essential suppressors involved in the $G_1$-S cell cycle transition (29), were remarkably increased in all cell lines after Aurora-A was knocked down (Aurora-Ai), and that cyclin-dependant kinase 4 (Cdk4) was decreased only in OVCA432 and OVCA433 cells, although its partner proteins, Cdk6 and cyclin D1, were not altered in any cell lines. The S-phase regulatory proteins cyclin E and its partner Cdk2 were not significantly changed in cells with or without Aurora-A shRNA. However, we found that cyclin B1, a $G_2$-M transition-promoting protein, was decreased in SKOV3 and OVCA433 cells after Aurora-A was silenced. These data suggest that Aurora-A promotes cell cycle progression in the $G_1$-S and $G_2$-M transitions, possibly through suppression of p21 and pRb, and that other factors, such as Cdk4 and cyclin B1, may also be involved in Aurora-A–associated

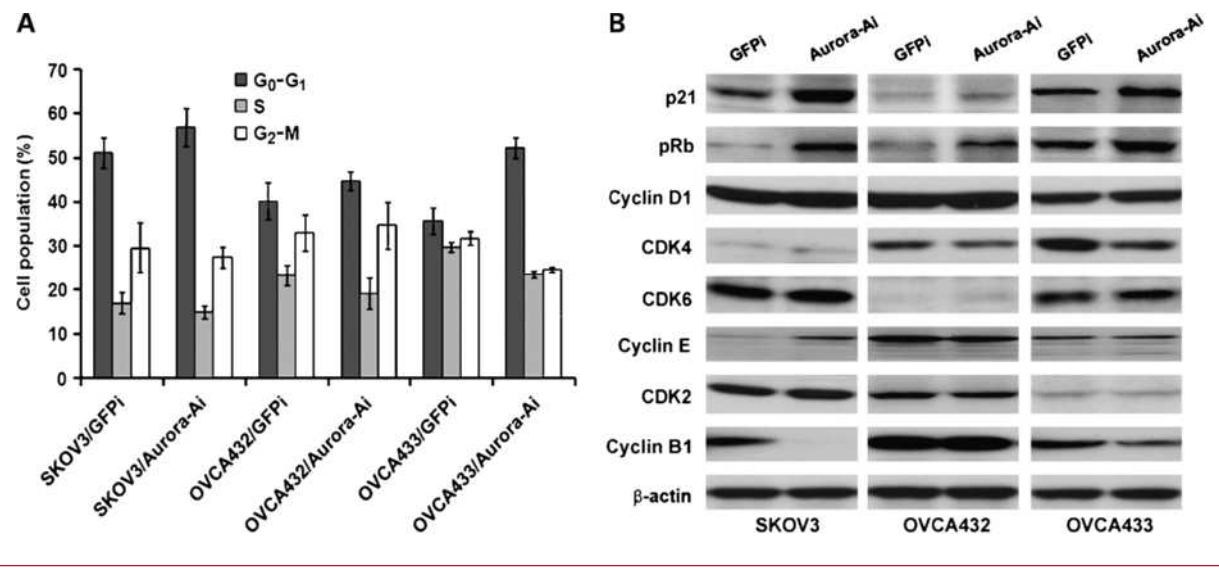


**Fig. 3.** Alteration of cell cycle associated with overexpression of Aurora-A. A, cell population was increased in $G_0$-$G_1$ phase and decreased in S phase in all cell lines treated with Aurora-A shRNA (Aurora-Ai) compared with that in controls (GFPi) but was decreased in the $G_2$-M phase only in SKOV3 and OVCA433 cells after Aurora-A was silenced. The scales (0 ~ 60%) represent the different population percentages of cells in different cell cycle phases. $G_0$-$G_1$ + S + $G_2$-M ≈ 95 ~ 100%. Error bars, 95% confidence interval. B, elevated expression of p21 and pRb, two cell cycle restrictors of the $G_0$-$G_1$-S transition, was observed after Aurora-A was silenced (Aurora-Ai), whereas Cdk4 and cyclin B1 were detected with limited reduction in two cell lines treated with Aurora-A shRNA (Aurora-Ai).

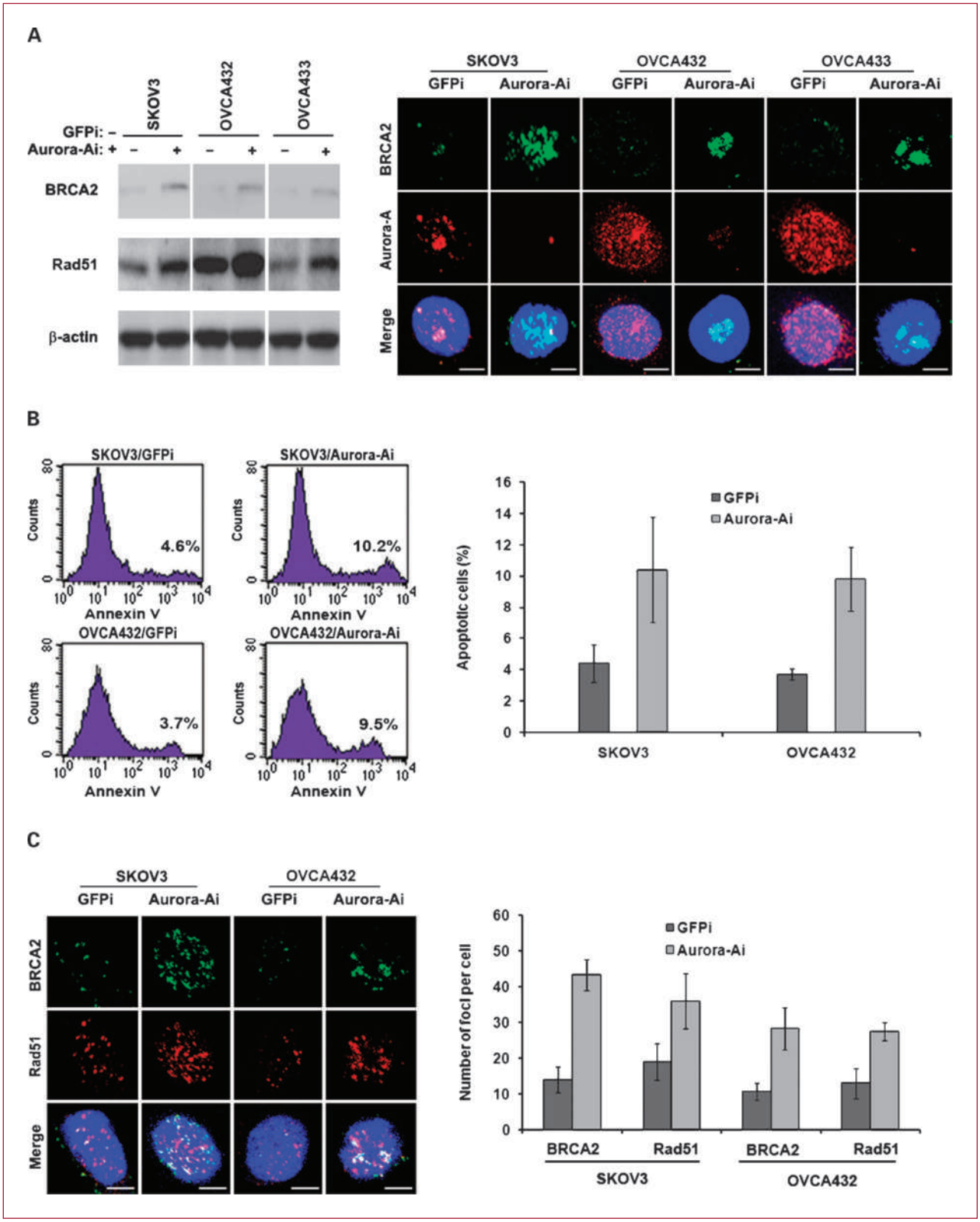


**Fig. 4.** Enhanced cell apoptosis and DNA damage response after silencing of Aurora-A. As the elevation of BRCA2 expression was detected by Western blotting and by immunoflourescence (A) after abrogation of Aurora-A by shRNA, the number of apoptotic cells was increased as indicated by Annexin V staining (B). The number of DNA damage repair foci for both BRCA2 and Rad 51 was increased (C) after γ-irradiation (10 Gy) in cells treated with Aurora-A shRNA (Aurora-Ai) which recovered the expression level of BRCA2 and Rad 51 (A). Error bars, 95% confidence interval.

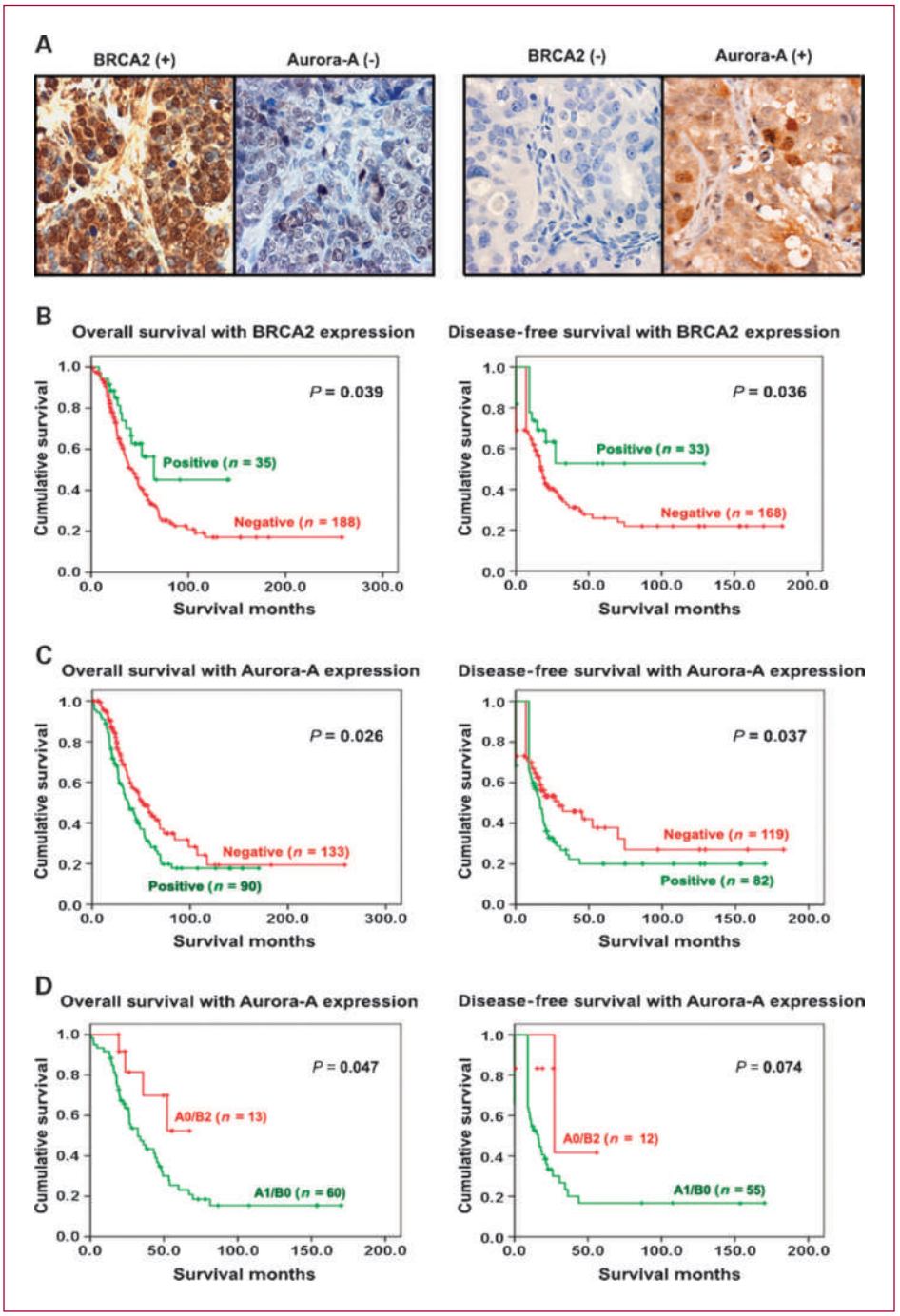


**Fig. 5.** Expression of BRCA2 and Aurora-A in high-grade ovarian serous carcinoma and correlation with patient survival. Representative images from tissue microarray stained for BRCA2 and Aurora-A. A, left, positive expression of BRCA2 in nuclei was correlated with negative expression of Aurora-A in the same core of high-grade ovarian carcinoma (×400). Right, positive Aurora-A expression was correlated with negative nuclear accumulation of BRCA2 in the same core of high-grade ovarian carcinoma (×400). B, favorable overall survival ($P$ = 0.039) and disease-free survival ($P$ = 0.036) were associated with positive nuclear accumulation of BRCA2. C, poor overall survival ($P$ = 0.026) and disease-free survival ($P$ = 0.037) were associated with positive expression of Aurora-A. D, an increased ratio of Aurora-A to BRCA2 (A1/B0) was associated with poor overall survival ($P$ = 0.047) but not disease-free survival ($P$ = 0.074) compared with a decreased ratio of Aurora-A to BRCA2 (A0/B2) that is correlated with favorable overall survival for ovarian cancer patients.

cell cycle progression depending on the genetic background of individual cell lines.

### Silence of Aurora-A enhances apoptosis and DNA damage repair through recuperation of BRCA2

A previous report showed that the amplification of Aurora-A is more common in breast cancer with *BRCA2* mutations (30). BRCA2 was also shown to be located in the centrosome (31) and to suppress polyploidy (32). Loss of BRCA2 expression may induce centrosome amplification and abnormal cell division (33). We suspected that loss of BRCA2 is associated with overexpression of Aurora-A, which leads to genomic instability and ovarian tumorigenesis. As shown in Fig. 4A, the expression level of BRCA2 was increased after Aurora-A was silenced, which was confirmed by immunofluorescent staining of Aurora-A and BRCA2 (Fig. 4A). In addition, the cell apoptosis in two cell lines tested was also increased at least by 2-fold

in the presence of Aurora-A shRNA compared with apoptosis in control cells (Fig. 4B). Moreover, the number of DNA damage repair foci composed with BRCA2 and Rad51 was increased after cells were treated with γ-irradiation (10 Gy; Fig. 4C). These results show that Aurora-A negatively regulates BRCA2 expression in ovarian cancer cells: overexpression of Aurora-A suppresses BRCA2-induced DNA damage repair and apoptosis, whereas knockdown of Aurora-A recovers BRCA2 expression, leading to increased cellular apoptosis and DNA damage response.

### Negative correlation between BRCA2 and Aurora-A predicts prognosis in patients with ovarian cancer

To investigate whether the negative regulation of BRCA2 by Aurora-A is associated with clinical significance in ovarian cancer, we did TMA staining using antibodies to Aurora-A or BRCA2 on tumor samples from a total of 223 patients with high-grade ovarian serous carcinoma. The nuclear score for BRCA2 was negatively correlated with total Aurora-A staining ($P$ = 0.024, two-tailed Pearson correlation) in 223 samples, as evidenced by the representative images showing either positive BRCA2 and negative Aurora-A expression (Fig. 5A, left) or negative BRCA2 and positive Aurora-A expression (Fig. 5A, right). The nuclear accumulation of BRCA2 was significantly associated with good overall survival (35 of 223 patients, or 15.7%; $P$ = 0.039) and disease-free survival (33 of 201 patients, or 16.4%; $P$ = 0.037; Fig. 5B). Strong staining for Aurora-A was significantly associated with poor overall survival (90 of 223 patients, or 40.3%; $P$ = 0.026) and disease-free survival (82 of 201 patients, or 40.8%; $P$ = 0.037; Fig. 5C). These data suggest that the expression of Aurora-A or BRCA2 can predict the outcomes of patients with high-grade serous ovarian cancer. We also analyzed the association between progression and the expression ratio between Aurora-A and BRCA2 in high-grade serous carcinomas. As shown in Fig. 5D, an increased ratio of Aurora-A to BRCA2 (A1 to B0) predicted poor overall survival ($P$ = 0.047, left) but not disease-free survival ($P$ = 0.074, right) compared with a low ratio of Aurora-A to BRCA2 (A0 to B2) that indicated a good overall survival for ovarian cancer patients. No statistical significance of outcomes was found between other ratios in terms of overall and disease-free survival (data not shown).

### Negative correlation between BRCA2 and Aurora-A in multiple cancer types

To examine whether the negative correlation between BRCA2 and Aurora-A represents a general mechanism in other human cancers, we did similar tissue microarray analyses on samples of human pancreas, breast, and colon cancers. Of 124 pancreatic cancer specimens, we found positive nuclear accumulation of BRCA2 in 51 samples (41.1%), which also showed negative expression of Aurora-A. Conversely, we found positive Aurora-A staining in 92 samples (74.2%), which also showed negative expression of BRCA2 (Supplementary Fig. S2A, images). There was a significant negative correlation ($P$ = 0.022, two-tailed Pearson correlation) between BRCA2 and Aurora-A. The Aurora A–positive cases are predominantly associated with negative expression of BRCA2 (Supplementary Fig. S2A, diagram, A1-3/B0), whereas BRCA2-positive cases are predominantly associated with negative expression of Aurora-A (Supplementary Fig. S2A, diagram, A0/B1-3; A1/B2-3). Of 208 breast cancer tissues, we found positive nuclear expression of BRCA2 in 80 samples (38.5%) and positive Aurora-A staining in 121 samples (58.2%; Supplementary Fig. S2B, images). There was also a significant negative correlation between BRCA2 and Aurora-A in breast cancer ($P$ = 0.003). Positive expression of Aurora A is associated with negative expression of BRCA2 (Supplementary Fig. S2B, diagram, A1-3/B0), whereas positive BRCA2 expression is predominantly associated with negative expression of Aurora-A (Supplementary Fig. S2B, diagram, A0/B1-3; A1/B1-3). Of 210 colon cancer tissues, we found positive nuclear BRCA2 staining in 140 samples (66.7%) and positive Aurora-A staining in 80 samples (38.1%; Supplementary Fig. S2C, images). There was a negative correlation between BRCA2 and Aurora-A in the colon cancer samples, although it did not reach statistical significance ($P$ = 0.135). Positive expression of Aurora A is associated with negative expression of BRCA2 (Supplementary Fig. S2C, diagram, A1-3/B0), whereas positive BRCA2 expression is predominantly associated with negative expression of Aurora-A (Supplementary Fig. 2C, diagram, A0/B1-3; A1/B1-3). These data show that the negative correlation between Aurora-A and BRCA2 exists in multiple cancer types, which may represent a general mechanism for Aurora-A–associated human cancer development.

## Discussion

Using ovarian cancer cell lines as a model, we have shown that overexpression of Aurora-A drives genomic instability and controls ovarian tumorigenesis by inducing centrosome amplification, multipolar spindle formation, and chromosome aberration. Aurora-A suppresses the expression of p21, pRb, and BRCA2 to advance cell cycle progression and to abolish cellular apoptosis and DNA damage response. The negative correlation between Aurora-A and BRCA2 exists in multiple cancer types, including pancreas and breast cancers, and the expression ratio of Aurora-A to BRCA2 can predict clinical outcomes for patients with ovarian cancer. Thus, our data provide a new insight into the mechanism of how Aurora-A signaling drives tumor development.

Aurora-A may regulate cell cycle checkpoint by modulating cell mitosis (34); little is known, however, about the downstream targets of Aurora-A in regulating the cell cycle. We have shown that Aurora-A may promote the $G_1$-S cell transition by suppressing p21 and pRb expression in ovarian cancer cells, given that knockdown of Aurora-A is able to restore the expression of p21 and pRb and to block the expression of the cell cycle progression factor Cdk4, leading to increased $G_0$-$G_1$ cell cycle arrest. In addition, the

increased p21 and pRb may also contribute to enhanced cell apoptosis (35, 36) and reduced genomic instability (37, 38).

The tumor suppression function of BRCA2 is mediated by multiple processes, including suppression of cell proliferation (39) and maintenance of DNA damage repair (40) and genomic integrity (41). Inactivation of BRCA2 induces genomic instability as a result of defective DNA damage repair and cell cycle dysregulation in cancer cells (42, 43). Our findings indicate that Aurora-A negatively regulates the expression of BRCA2 to control genomic instability in ovarian cancer cells, as shown by our result that knockdown of Aurora-A can restore BRCA2 expression, leading to increased genomic stability and decreased tumorigenesis. The clinical correlation between Aurora-A and BRCA2 and patient survival strongly suggests that the negative correlation illustrated by results showing that the expression ratio of Aurora-A and BRCA2 can be used to predict ovarian cancer outcomes. We found that the distribution of BRCA2 expression was lower in ovarian cancer (12.5%) and breast cancer (38.5%) than in pancreatic cancer (41.1%) and colon cancer (66.7%), suggesting that inactivation of BRCA2 in ovarian and breast cancer is more frequent than in pancreatic and colon cancer. The reverse correlation between BRCA2 and Aurora-A was also found in pancreatic cancer and breast cancer and thus may represent a general mechanism in epithelial cancers with overexpression of Aurora-A.

Although we have shown, to our knowledge for the first time, that Aurora-A represses the expression of p21, pRb, and BRCA2 in ovarian cancer cells, whether this phenomenon is regulated directly by Aurora-A remains unknown. It is well known that Aurora-A suppresses the tumor suppressor p53 in various tumor cells (44, 45) and mediates its degradation by phosphorylation (8, 9, 46). It is also known that p53 regulates p21 and pRb to control cell cycle arrest and apoptosis in different cancer cells (47). Therefore, whether the repression of p21 and pRb by Aurora-A is mediated through p53 remains to be further investigated. The most valuable finding in our study is that Aurora-A represses BRCA2 expression, but whether the suppression of BRCA2 expression is mediated directly by Aurora-A at the transcriptional or posttranslational level is unclear. One of the most likely mechanisms, based on emerging evidence, is that the stable expression of BRCA2 largely depends on proteasome-mediated ubiquitination and degradation in many cancers by interacting with multiple gene products such as USP11 (a deubiquitinating enzyme; ref. 48), Skp2 (a subunit of the Skp1-Cul1-F-box protein ubiquitin complex; ref. 49), and cancer-associated BRAD1 β (50). However, whether this proteasome-mediated ubiquitination and degradation of BRCA2 is triggered by Aurora-A in a specific manner such as phosphorylation of BRCA2 requires further study.

In summary, our data show that overexpression of Aurora-A represses p21, pRb, and BRCA2, which promotes cell cycle progression, antiapoptosis, and genomic instability, leading to increased tumorigenesis. The negative correlation between Aurora-A and BRCA2 may represent a novel prognostic marker for ovarian cancer and a generalized mechanism in cancer development.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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