

Overexpression of the Centrosomal Protein Aurora-A Kinase is Associated with Poor Prognosis in Epithelial Ovarian Cancer Patients

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Abstract Purpose: To assess the clinical significance of Aurora-A kinase, a centrosome-regulating serine-threonine kinase, in ovarian carcinoma.

Experimental Design: Aurora-A kinase expression was assessed by Western blot (cell lines) or immunohistochemistry (high-grade epithelial ovarian cancers), and clinical variables were collected by retrospective chart review. Centrosome amplification was assessed by immunofluorescence in cell lines, and by immunohistochemistry in patient samples.

Results: All ovarian cancer cell lines exhibited significant Aurora-A kinase protein overexpression, and all except A2780-par had centrosome amplification, a characteristic of mitotic dysregulation leading to genomic instability. Fifty-eight of 70 patient samples (82.8%) exhibited Aurora-A kinase overexpression compared with normal ovarian surface epithelium. High Aurora-A kinase expression was strongly associated with supernumerary centrosome count in tumor cells ($P < 0.001$). Tumors with the greatest Aurora-A overexpression ($n = 24$) had decreased patient survival (median survival, 1.44 versus 2.81 years; $P = 0.01$). High Aurora-A expression and suboptimal surgical cytoreduction remained predictors of poor survival ($P < 0.05$) by multivariate analysis.

Conclusions: Aurora-A kinase is overexpressed by a substantial proportion of ovarian cancers and is associated with centrosome amplification and poor survival. It may be a useful prognostic marker and target in ovarian cancer.

The stepwise process of mitosis involves checkpoints that are highly regulated. Included in this process are the maturation and separation of centrosomes, which participate in spindle assembly, chromosomal separation, and equal partitioning into daughter cells, as required for genomic stability. Aurora-A (also known as *STK15* and *BTAK*) is a serine-threonine kinase that is essential to proper centrosome functions (1–3). Up-regulation

of Aurora-A is frequently noted in tumors at the mRNA, protein, and chromosomal (site 20q13.2) level (1, 2, 4–11). Moreover, induced amplification of Aurora-A kinase is capable of converting benign fibroblasts into a malignant phenotype (1, 2). Aurora-A polymorphisms have been described as conferring an 18% to 25% increased risk of developing ovarian cancer (12), consistent with a reported 22% to 59% increased risk of breast or colon cancer with the *STK15* + 91A polymorphism (13). Thus, Aurora-A kinase seems to play an important role in malignant transformation, and represents a novel target for therapy (14).

In ovarian cancer, Aurora-A kinase is amplified in many ovarian cancer cell lines (15), and is overexpressed during ovarian epithelial cell transformation (16). This may be due to gene amplification, as the site of the Aurora-A kinase gene is frequently amplified in ovarian cancer (7). One analysis of patient samples from a relatively heterogeneous population found that 57% of ovarian tumors exhibited high Aurora-A expression (9). A relationship is also suggested by the finding that the chromosomal region 20q12–23, the location of the Aurora-A kinase gene, was amplified in the majority of ovarian cancer cell lines and tumors (7, 9). However, the clinical and functional significance of Aurora-A kinase in ovarian carcinoma is not fully known. Therefore, we examined several ovarian cancer cell lines for Aurora-A kinase expression and centrosome amplification. Furthermore, a cohort of advanced stage high-grade epithelial ovarian cancer patient samples were tested for both Aurora-A expression and centrosome amplification, and

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its expression correlated with survival and other clinical characteristics. We have found that ovarian cancer patients with increased tumoral Aurora-A expression show a higher incidence of supernumerary centrosomes, as well as poor survival, suggesting that this kinase may not just be associated with cancer initiation, but with progression as well.

Materials and Methods

Ovarian cancer cell lines and centrosome amplification assessment.

For immunofluorescence staining of centrosomes, ovarian cancer cell lines SKOV3, EG, A2780-par, 222, OVCAR3, HeyA8, and HeyA8-MDR (17, 18) and the non-transformed ovarian cell line H10-180 (a kind gift from Dr. Andrew Godwin, Fox Chase Cancer Center, Philadelphia, PA) were grown in monolayers on poly-D-lysine-coated glass coverslips, and permeabilized in 0.5% Triton X-100 in PEM buffer [80 mmol/L PIPES (pH 7.0), 5 mmol/L EGTA, 2 mmol/L MgCl₂] at room temperature. Cells were fixed in 3.7% paraformaldehyde in PEM buffer at room temperature for 15 min. The cells were washed thrice in PEM buffer for 5 min at room temperature and blocked with 5% nonfat milk for 1 h at room temperature before being incubated with an Aurora-A rabbit polyclonal antibody (diluted 1:1,000; Bethyl Labs) in 5% nonfat milk in PBST (PBS with 0.5% Tween 20) at room temperature for 1 h. Cells were washed thrice in PBST buffer and treated with a goat anti-rabbit secondary antibody FITC-conjugated (Alexa-488; Molecular Probes) for 1 h at room temperature. The coverslips were washed thrice in PBST buffer, counterstained with 4',6-diamidino-2-phenylindole and inverted onto clean glass slides with mounting media (Molecular Probes, Inc.). Images were collected on a Zeiss Axiovert deconvolution fluorescence microscope. The number of cells with more than two centrosomes was counted (~400 cells counted).

Western blot. Cultured cell lysates were prepared by washing cells with PBS followed by incubation in modified radioimmunoprecipitation assay lysis buffer [50 mmol/L Tris, 150 mmol/L NaCl, 1% Triton, 0.5% deoxycholate plus 25 µg/mL leupeptin, 10 µg/mL aprotinin, 2 mmol/L EDTA, and 1 mmol/L sodium orthovanadate (Sigma Chemical Co.)] for 10 min at 4°C. Cells were scraped from plates, centrifuged at 13,000 rpm for 20 min at 4°C, and the supernatant stored at -80°C. Protein concentrations were determined using a bicinchoninic acid protein assay reagent kit (Pierce Biotechnology), and subjected to 10% SDS-PAGE separation. For Western blot, samples transferred to a nitrocellulose membrane by semidry electrophoresis (Bio-Rad Laboratories) were incubated with 1.0 µg/mL of anti-Aurora-A rabbit polyclonal antibody (Bethyl Laboratories) for 1 h at room temperature, detected with 1.0 µg/mL of horseradish peroxidase-conjugated anti-rabbit IgG (Amersham), and developed using an enhanced chemiluminescence detection kit (ECL, Pierce). Membranes were tested for equal loading by probing for β-actin, 0.1 µg/mL anti-β-actin primary antibody (Sigma).

Patient samples. After approval by the M.D. Anderson Cancer Center Institutional Review Board for the Protection of Human Subjects, 70 formalin-fixed, paraffin-embedded human ovarian cancer samples and 5 normal ovarian surface epithelium samples were collected. All of the patients underwent surgical exploration and cytoreduction as the initial treatment. The treating gynecologic oncologist determined the adjuvant therapy. Diagnosis was verified by a pathology review at the institutional gynecologic oncology tumor board. All of the patients were staged according to the International Federation of Gynecology and Obstetrics surgical staging system. A gynecologic pathologist reviewed all of the pathology results for all of the patients.

Immunohistochemistry for Aurora-A kinase and centrosomes. Formalin-fixed, paraffin-embedded sections were deparaffinized by sequential washing with xylene, 100% ethanol, 95% ethanol, 80% ethanol, and PBS. For antigen retrieval, slides incubating in 0.1 mol/L of sodium citrate in dH₂O (pH 6.0) were heated in a steam cooker for 20 min. Endogenous peroxidases were blocked with 3% H₂O₂ in methanol for

10 min. After washing with PBS, slides were blocked with 5% normal horse serum and 1% normal goat serum in PBS for 15 min at room temperature, followed by incubation with 1 mg/mL of rabbit anti-human Aurora-A kinase antibody (Bethyl Laboratories) in blocking solution overnight at 4°C. After washing twice with PBS, slides were incubated with a biotinylated anti-rabbit secondary antibody conjugated (30 min) and streptavidin/horseradish peroxidase (30 min; Dako), followed by 3,3'-diaminobenzidine (Phoenix Biotechnologies) substrate for 5 min, washed with water, and counterstained with Gil No. 3 hematoxylin (Sigma) for 20 s. To confirm that the anti-Aurora antibody was specific and without cross-reactivity, additional samples were tested by peptide neutralization. Immunohistochemistry was done as above, with the exception that separate slides were incubated

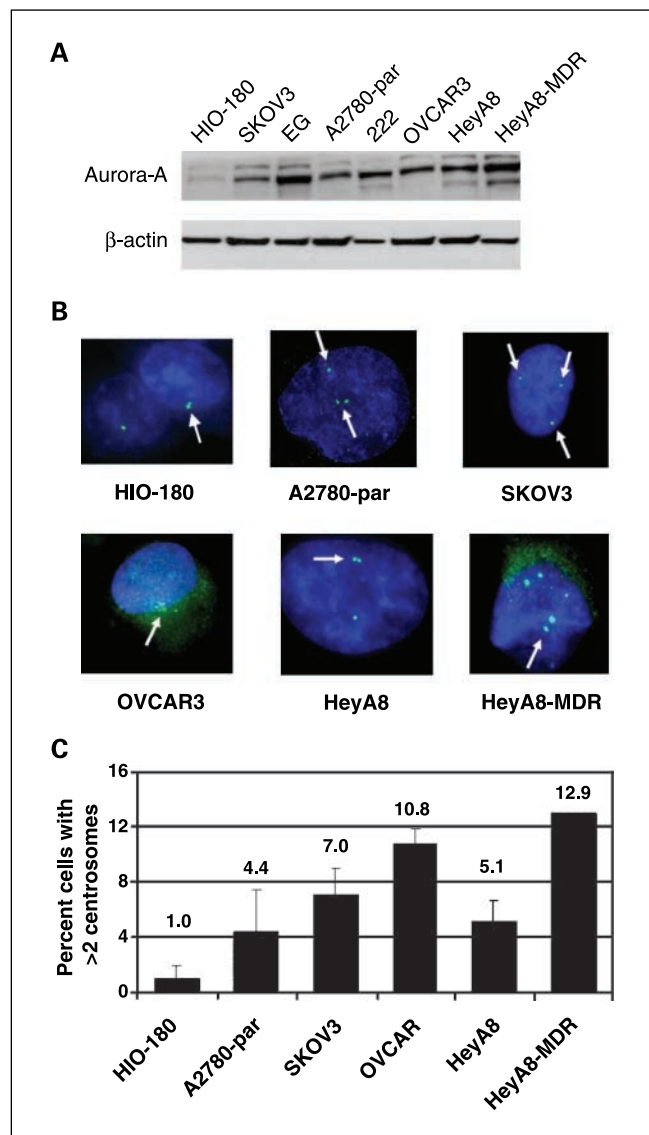


Fig. 1. Aurora-A kinase expression in ovarian cancer cell lines. **A**, whole cell lysates from multiple ovarian cancer cell lines and H10-180 were subjected to Western blot using primary anti-Aurora-A kinase antibody. **B**, cells in culture were fixed in acetone, and exposed to anti-centrosome antibodies. After secondary antibody-FITC (green), cells were exposed to 4',6-diamidino-2-phenylindole to stain nuclei blue and examined by fluorescent microscopy. White arrows, centrosomes. **C**, after fluorescent staining with anti-centrosome antibodies, the number of cells with more than two centrosomes was counted (at least 400 cells counted) and compared between cell lines. Normal cells have one to two identifiable centrosomes depending on cell cycle stage, whereas cancer lines with supernumerary centrosomes in >5% of cells were considered aneuploid (4).

Table 1. Patient characteristics

Characteristic	Incidence, n (%)
Age	28-89 (median, 62)
Stage	
III	55 (79)
IV	15 (21)
Cytoreduction	
Optimal	31 (44)
Suboptimal	36 (52)
Unknown	3 (4)
Histology	
Serous	64 (91)
Other	6 (9)
Grade	
High	70 (100)
Total no. of chemotherapy regimens	
1	22 (32)
2	19 (27)
3	15 (21)
>3	14 (20)

overnight at 4°C in either primary Aurora-A antibody (1:600), Aurora-A antibody (1:600) + Aurora-A-specific peptide block (Bethyl Laboratories, 1:600), primary antibody (1:600) + peptide block (1:300), or peptide block alone (1:600).

All of the samples were reviewed by a board-certified pathologist (M.T. Deavers) and gynecologic oncologist (C.N. Landen), who were blinded to the clinical outcome of these patients. Aurora-A expression was determined semiquantitatively by assessing the percentage of stained tumor cells and the staining intensity. The percentage of positive cells was rated as follows: 0 points, 0% to 5%; 1 point, 6% to 25%; 2 points, 26% to 50%; 3 points, 51% to 75%; 4 points, >75%. The staining intensity was rated as follows: 0 points, no staining; 1 point, weak intensity (equivalent to normal epithelium); 2 points, moderate intensity; 3 points, strong intensity. Points for expression and percentage of positive cells were added (total score of 0-7), and an overall score of 0 (total 0-1), 1 (total 2-3), 2 (total 4-5), or 3 (total 6-7) was assigned.

To determine if a correlation between Aurora-A kinase and centrosome amplification could be seen in ovarian cancer patient samples, the nine highest and nine lowest expressers of Aurora-A kinase were subjected to immunohistochemistry for identification of centrosomes. Deparaffinized tumor specimens were subjected to antigen retrieval in 10 mmol/L of citrate buffer (pH 6.0) in a pressure cooker for 10 min. Endogenous peroxidase was quenched with 3% H₂O₂, slides were blocked with 5% normal goat serum for 15 min, followed by incubation with mouse anti-human γ -tubulin antibody to identify centrosomes (200 μ g/mL; Santa Cruz, Inc.) for 1 h at room temperature. Slides were rinsed thrice in TBS-20 [10 mmol/L TBS (pH 7.6) + 0.5% Tween 20] and treated with the ELISA (EnVision, Dako) for 30 min. After washing thrice in TBS-20, amplification was done with 3,3'-diaminobenzidine (Dako) for

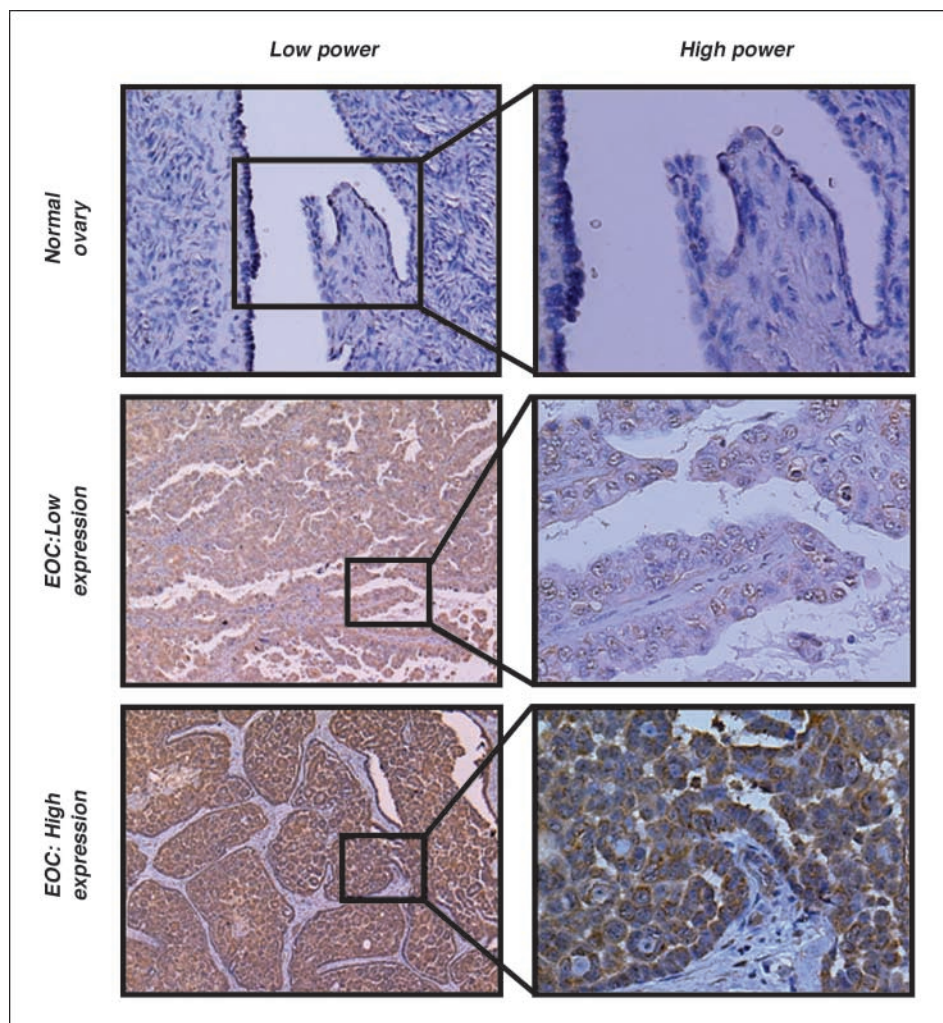


Fig. 2. Immunohistochemistry for Aurora-A kinase. Five normal ovaries and 70 epithelial ovarian cancer (EOC) specimens were stained for Aurora-A kinase. Examples of high and low staining in cancer specimens, and degree of staining in normal ovaries, are shown. Original magnification, $\times 100$ (low power) or $\times 400$ (high power).

15 min, and washed six times in water. The chromagen signal was enhanced by applying 3,3'-diaminobenzidine sparkle (Biocare) for 10 min, washed six times in water, and counterstained with Harris hematoxylin (Thermo-Shandon) for 30 s. Centrosome structures were identified and enumerated in four randomly selected fields of stained tissue sections using Axioplan microscope. Cells with more than two centrosomes per cell were counted in 200 consecutive cells and an average calculated among the nine samples in each group. The investigator counting the number of centrosomes (A. Immaneni) was blinded to Aurora-A expression.

Statistical analysis. The χ^2 test was used to determine differences among variables. Kaplan-Meier survival plots were generated, and comparisons between survival curves were made with the log-rank statistic. The Cox proportional hazards model was used for multivariate analysis. SPSS software was used (SPSS Inc.), and $P < 0.05$ was considered statistically significant.

Results

Aurora-A kinase expression and centrosome amplification in cell lines. Western blot analysis was conducted on multiple ovarian cancer cell lines for Aurora-A kinase protein expression. All cancer cell lines expressed high levels of Aurora-A protein (Fig. 1A), and expression levels were significantly higher

compared with HIO-180 cells, which are nontransformed immortalized human ovarian epithelial cells.

Because of the association of Aurora-A kinase overexpression and centrosome amplification, these same cell lines were then examined for supernumerary centrosomes. After immunofluorescent staining (Fig. 1B), the number of visible centrosomes was counted, and the percentage of cells with more than two centrosomes per cell was calculated (Fig. 1C). Cell lines were considered aneuploid if $>5\%$ of cells exhibit more than two centrosomes per cell (4), and all ovarian cancer cell lines exhibited supernumerary centrosomes. Interestingly, the HeyA8-MDR (multidrug-resistant) line is more aggressive and chemoresistant compared with its parental HeyA8 line, and its Aurora-A expression and average centrosome count were significantly higher than the parental line. In contrast, the non-transformed immortalized HIO-180 cell line had the lowest Aurora-A expression and a normal centrosome count, and the poorly tumorigenic A2780-par line showed moderate Aurora-A expression but a normal centrosomal count.

Aurora-A kinase is frequently overexpressed in human ovarian cancers. Immunohistochemistry was done on 70 paraffin-embedded samples obtained at the time of primary cytoreductive surgery, and 5 normal ovarian tissue samples. The clinical

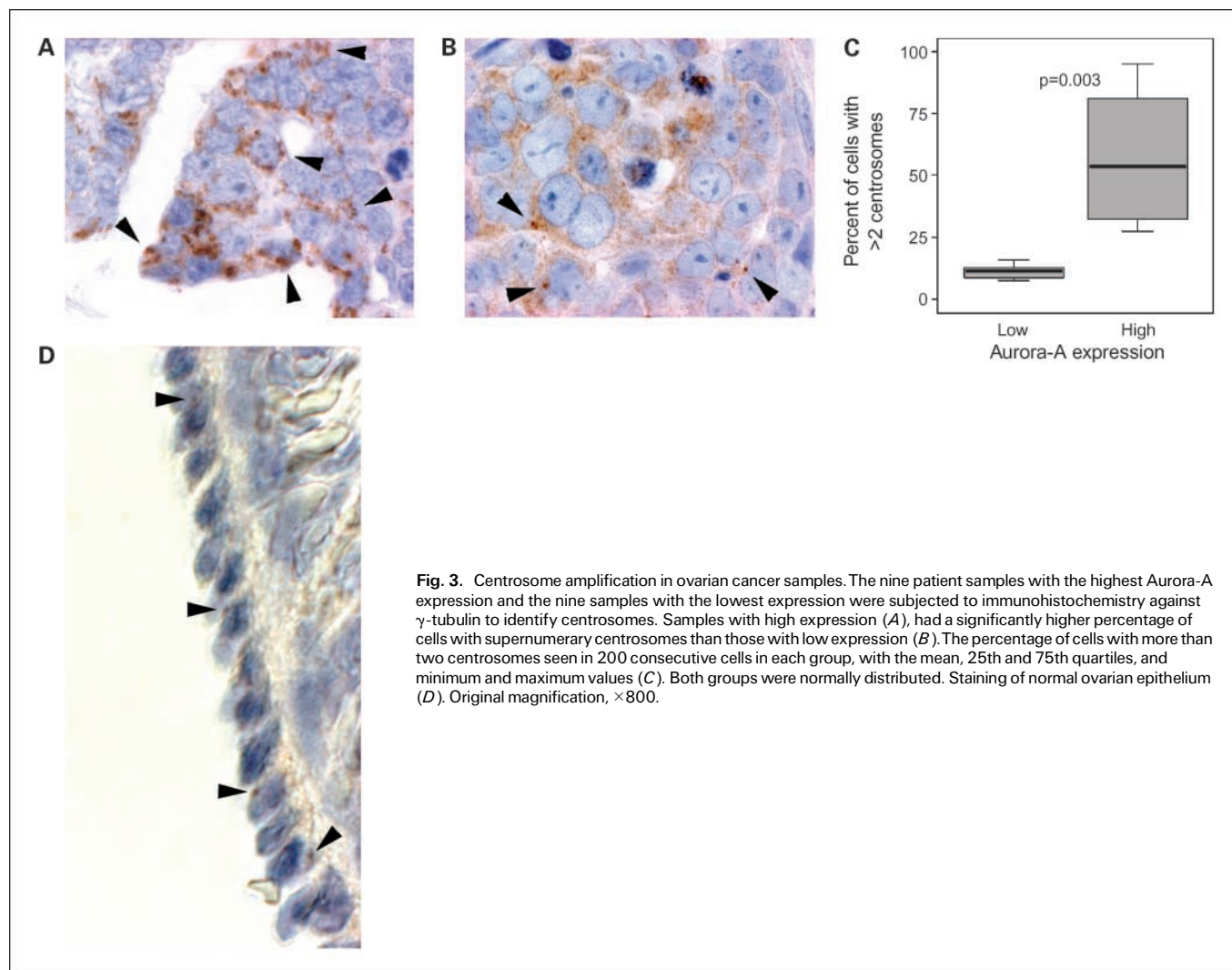


Table 2. Association of Aurora-A expression and clinical characteristics

	Aurora-A expression		High Aurora-A (%)	P
	High (n = 24)	Moderate/low (n = 46)		
Stage				
III	20	35	36.4	0.74
IV	4	11	26.7	
Cytoreduction*				
Optimal	10	21	32.3	0.92
Suboptimal	11	25	30.6	
Ascites				
Yes	17	41	29.3	0.12
No	7	5	58.3	

*Three patients had unknown cytoreduction status, all of which were in the high Aurora-A expression group.

characteristics of the 70 cancer patients were obtained by retrospective chart review, and are reviewed in Table 1. All patients had stage III to IV ovarian cancer and underwent cytoreductive surgery followed by taxane and platinum chemotherapy. All were of grade 3 serous histology. Cytoreduction was optimal (<1 cm residual disease) in 44.3% of patients.

Aurora-A staining was graded based on intensity and distribution of staining, and a composite score of 0 to 3 was assigned, as described in Materials and Methods. Representative high-power and low-power sections are presented in Fig. 2. The specificity of Aurora-A staining was confirmed by peptide neutralization, after which no significant signal could be seen (Supplementary Fig. S1). Staining was either weak (n = 2) or negative (n = 3) in all normal specimens. Staining intensity greater than that of normal ovarian epithelium (grade 2-3) was seen in 66% of cases, with the distribution of staining noted to be greater than half the tumor in 67% of cases. Both intensity and distribution were considered as factors in giving an overall staining score (as described in Materials and Methods). All normal samples had a score of 1, whereas a score of 1, 2, or 3 was given to 17%, 49%, and 34% of cancer samples, respectively. Therefore, expression was greater than that of normal epithelium in 82.9% of cases.

Aurora-A protein overexpression is associated with centrosome amplification. Aurora-A kinase overexpression may contribute to carcinogenesis by centrosome amplification, leading to abnormal mitosis, chromosomal missegregation, and promotion of aneuploidy. Centrosome amplification has been observed in cells in which Aurora-A kinase overexpression was extrinsically induced (19), but we sought to correlate Aurora-A expression and centrosome amplification in the same human samples. To determine if an association between centrosome amplification and Aurora-A kinase overexpression could be seen, centrosomes were identified by immunohistochemistry against γ -tubulin in the nine patient samples with the highest Aurora-A kinase expression (as in Fig. 3A), and in the nine patient samples with the lowest expression (as in Fig. 3B). Centrosome amplification was defined as more than two centrosomes in any one cell. In patients with the highest Aurora-A expression, the mean number of cells with centroso-

mal amplification was 52.4% (SD 27.4%), significantly higher than in patients with low expression (12.7%, SD 5.5%; P = 0.003; Fig. 3C). Staining of normal ovarian epithelium with zero to one identifiable centrosomes per cell is shown in Fig. 3D.

Aurora-A protein overexpression is associated with clinical features. An analysis of clinical factors and their association with Aurora-A kinase expression was conducted (Table 2). Aurora-A kinase expression was not significantly different when comparing stage III and IV patients, or the rate of optimal cytoreduction. Patients with ascites were less likely to have tumors with high Aurora-A kinase expression, but the difference was not significantly different (P = 0.12).

Survival among those patients with the strongest Aurora-A kinase staining in the tumor (overall score of 3, n = 24) was compared with those with low or moderate staining. The Kaplan-Meier curve is shown in Fig. 4. By univariate analysis, high Aurora-A kinase expression was associated with poor survival, with a median survival of 1.44 versus 2.81 years (P = 0.01, log-rank statistic). Finally, a multivariate analysis with the Cox proportional hazards model was conducted incorporating the variables stage, grade, optimal cytoreduction, and Aurora-A kinase expression (Table 3). Both optimal cytoreduction and Aurora-A kinase remained significant predictors of poor outcome (P < 0.005). These data suggest that Aurora-A kinase may be an independent predictor of survival in ovarian cancer.

Discussion

The key findings from this study are that Aurora-A kinase is overexpressed in most epithelial ovarian cancer cell lines and tumors. Although previous authors have described Aurora-A expression and centrosome amplification separately, we have shown that cell lines and patients with the highest Aurora-A overexpression have concurrent significantly higher centrosome amplification, strengthening this association. Centrosome

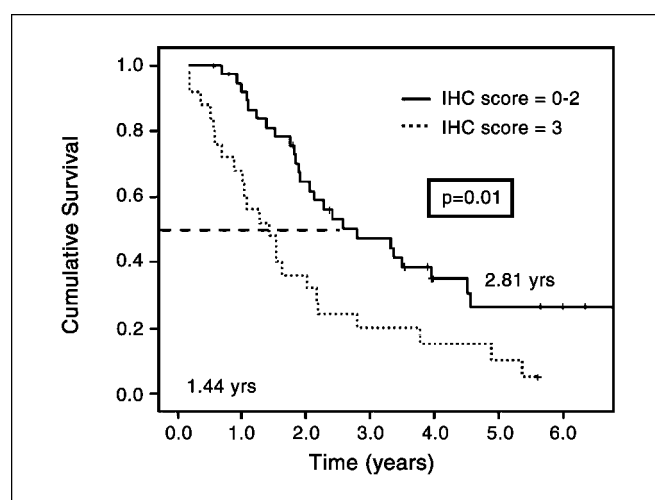


Fig. 4. Survival in patients with high Aurora-A expression. Overall survival in patients with the highest Aurora-A kinase immunohistochemistry scores (score = 3, n = 24) versus the remaining patients was plotted by the Kaplan-Meier method. Statistical comparison of survival between groups with the log-rank statistic suggests that patients with the highest Aurora-A overexpression in the tumor had poor survival compared with moderate or poor expression (P = 0.01).

Table 3. Multivariate analysis of clinical variables

Clinical characteristics	P
Stage III vs. IV	0.13
Ascites	0.33
Cytoreduction	0.001
Aurora-A overexpression	0.005

amplification is a mechanism that can lead to chromosomal instability, which in turn, results in further mutations. Therefore, Aurora-A kinase overexpression may result in genetic instability that may not only predispose to the rapid development of epithelial ovarian cancer, but also predispose cells to further genetic alterations and heterogeneity with evolving resistance to therapy. Consistent with this, increased Aurora-A kinase expression was strongly related to centrosome amplification and poor patient survival. This suggests that Aurora-A kinase may not only be involved in the initiation of cancer, but also in the progression, and therefore might be a reasonable target for directed therapeutics.

Many mechanisms by which Aurora-A kinase amplification may promote malignancy have been proposed. In a rat mammary tumor model, alteration of Aurora-A is an early event, suggesting a role in transformation (20). When benign human ovarian epithelial cells are transformed in a stepwise fashion *in vitro*, there is a progressive increase in Aurora-A kinase expression (16). Induced Aurora-A overexpression alone, in p53^{+/−} mice, leads to the development of mammary tumors in 70% of mice, with concurrent genetic instability, chromosomal defects, and Akt activation (21). Furthermore, ectopic Aurora-A overexpression induces telomerase activity through increased hTERT production (22). Some method of telomere maintenance is required for cell immortalization in all cancers, and telomerase expression through increased hTERT production is a frequent characteristic of ovarian cancer (23).

Regarding a genetic instability mechanism, Aurora-A overexpression promotes tetraploidization, a precursor to aneuploidy (24). If the normal G₂ checkpoint that identifies aneuploidy and induces apoptosis is defective, further cellular division and chromosome instability results, promoting tumorigenesis (3, 25). Aurora-A polymorphisms lead to aberrant mitosis and aneuploidy (12). Aurora-A has also been shown to interact with p53 and reduce its effectiveness as a protector of genome integrity (26, 27). We have described a direct correlation between Aurora-A expression and centrosome amplification, which provides a clear pathway towards the development of aneuploidy.

If Aurora-A kinase were only involved in promoting the formation of tumors, targeting this protein would only have

benefit in cancer prevention, not treatment. However, there is growing evidence that Aurora-A kinase also plays a role in cancer progression and resistance to therapy. One study found that Aurora-A overexpression was more often found in early stage and low-grade ovarian cancers, suggesting that it was an early event in carcinogenesis (9). However, more recent studies indicate that it may also contribute to tumor aggressiveness. The association of Aurora-A overexpression with increased invasiveness of esophageal malignancies has been shown (10), as has higher grade, stage, and/or mortality in breast and hepatocellular carcinoma (28, 29). Aurora-A protects cells from chemotherapy-induced apoptosis by activation of Akt, conferring resistance to cisplatin, etoposide, and paclitaxel (30). Multiple studies have shown that Aurora-A overexpression disrupts cell cycle checkpoint functions (25), leading to an increase in proliferation. Aurora-A overexpression disrupts the spindle checkpoint, so that defective spindle formations induced by paclitaxel are inconsequential, making cells paclitaxel-resistant (31). Centrosome hyperamplification correlates with chromosomal instability and tumor aggressiveness in multiple tumor types (32, 33). Furthermore, increased tumor aneuploidy promotes genetic instability, resulting in greater heterogeneity and chance of treatment-resistant clones existing in tumors. Targeting Aurora-A to reduce expression may overcome these tumor-promoting effects.

The recognition of the role of Aurora-A in carcinogenesis has led to the development of promising small molecule inhibitors (34). These include VX-680/MK-0457, which is a potent inhibitor of Aurora-A kinase and blocks cell cycle progression, induces apoptosis, and inhibits tumor growth in a variety of tumor cell types and xenograft models (35). Targeting Aurora-A kinase for down-regulation with RNA interference methods showed reduced *in vitro* proliferation and *in vivo* tumorigenicity in mice (36). In this study, pancreatic cancer cells with decreased Aurora-A were more sensitive to paclitaxel. Synergistic activity with paclitaxel was believed to be due to a dual targeting mechanism of arresting cells in the G₂-M phase of the cell cycle (36). RNA interference-induced down-regulation of Aurora-A kinase has also been shown to suppress migration in esophageal cancer cells (10). Recently, a feasible method for the delivery of small interfering RNA *in vivo* using liposomal packaging has been shown and may allow an alternative strategy for Aurora-A kinase targeting (37).

In conclusion, we have shown that the Aurora-A kinase oncoprotein, which seems to play a role in both tumor initiation and progression, is frequently overexpressed in ovarian cancer cell lines and human tumor specimens. Aurora-A kinase overexpression is associated with centrosome amplification, and patients with the highest tumoral levels of Aurora-A have poor clinical outcomes. Examination of Aurora-A targeting agents in ovarian cancer is warranted.

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