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...
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, EC, 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-o-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 in PBST buffer [5% nonfat milk in PBS with 0.5% Tween 20] at room temperature before being incubated with an Aurora-A rabbit polyclonal antibody (diluted 1:1,000; fetal Bovine) 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 radiolabeled precipita-tion 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 bicinecinic 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 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 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.

Aurora-A Kinase Expression and Centrosome Amplification

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 45% of cells were considered aneuploid (4).
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).

Table 1. Patient characteristics

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

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 μg/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, ×100 (low power) or ×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 χ² 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

<table>
<thead>
<tr>
<th>Stage</th>
<th>High Aurora-A (%)</th>
<th>Moderate/low Aurora-A (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 24)</td>
<td>(n = 46)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>20</td>
<td>35</td>
<td>36.4 0.74</td>
</tr>
<tr>
<td>IV</td>
<td>4</td>
<td>11</td>
<td>26.7</td>
</tr>
<tr>
<td>Cytoreduction*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optimal</td>
<td>10</td>
<td>21</td>
<td>32.3 0.92</td>
</tr>
<tr>
<td>Suboptimal</td>
<td>11</td>
<td>25</td>
<td>30.6</td>
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<tr>
<td>Ascites</td>
<td>Yes</td>
<td>17</td>
<td>29.3 0.12</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>7</td>
<td>58.3</td>
</tr>
</tbody>
</table>

*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 = 3) 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 staining score (as described in Materials and Methods). All distributions 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 = 3) 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 expression, the mean number of cells with centrosome 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 concurrently higher centrosome amplification, strengthening this association. Centrosome
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 tetraploidy, 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 kinase overexpression is associated with centrosome amplification, and patients with the highest tumoral levels of Aurora-A kinase overexpression are associated with centrosome amplification, and patients with the highest tumoral levels of Aurora-A kinase overexpression are more likely to have a poor outcome (9). Recent studies indicate that Aurora-A kinase promotes telomere packaging has been shown and may allow an alternative 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 oncprotein, 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 kinase overexpression are associated with a poor clinical outcome.

References

Table 3. Multivariate analysis of clinical variables

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<tr>
<th>Clinical characteristics</th>
<th>P</th>
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<tr>
<td>Stage III vs. IV</td>
<td>0.13</td>
</tr>
<tr>
<td>Ascites</td>
<td>0.33</td>
</tr>
<tr>
<td>Cytoreduction</td>
<td>0.001</td>
</tr>
<tr>
<td>Aurora-A overexpression</td>
<td>0.005</td>
</tr>
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