Activation and Overexpression of Centrosome Kinase BTAK/Aurora-A in Human Ovarian Cancer

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ABSTRACT

Previous studies have demonstrated amplification of the centrosome serine/threonine kinase BTAK/Aurora-A in 10–25% of ovarian cancers. However, alterations of BTAK/Aurora-A at kinase and protein levels and its role in ovarian cancer progression have not been well documented. In this study, we examined the kinase activity and protein levels of BTAK/Aurora-A in 92 patients with primary ovarian tumors. In vitro kinase analyses revealed elevated BTAK/Aurora-A kinase activity in 44 cases (48%). Increased BTAK/Aurora-A protein levels were detected in 52 (57%) specimens. High protein levels of BTAK/Aurora-A correlated well with elevated kinase activity. Activation and overexpression of BTAK/Aurora-A were more frequently detected in early stage/low-grade ovarian tumors, although there was no statistically significant association at the kinase level between early stage/low-grade and late stage/high-grade tumors. Moreover, BTAK/Aurora-A was preferentially expressed in noninvasive tumors, as revealed by immunohistochemical staining, suggesting that alterations of BTAK/Aurora-A could be an early event in human ovarian oncogenesis. To our knowledge, this is the first demonstration of recurrent activation and overexpression of BTAK/Aurora-A in human ovarian cancer, which may play a critical role in development of this malignancy.

INTRODUCTION

BTAK/Aurora-A (also known as STK15, aurora-2, ARKI, and AIKI) is a serine/threonine protein kinase that belongs to the

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Clinical Cancer Research
BTAK/Aurora-A at the kinase and protein levels are common events, which could play a pivotal role in human ovarian oncogenesis.

**MATERIALS AND METHODS**

**Tumor Specimens, Cell Lines, Transfection, and Statistical Analysis.** All of the primary human ovarian cancer specimens were obtained from patients who underwent surgery at H. Lee Moffitt Cancer Center, and each sample contained \( \geq 80\% \) tumor cells, as was confirmed by microscopic examination. The tissues were snap frozen and stored at \(-70^\circ C\). Histopathologically, the ovarian cancer specimens include 10 LMP, 58 serous, 12 mucinous, and 6 endometrioid ovarian surface epithelial cystadenocarcinomas, 1 clear cell carcinoma, and 1 mix tumor. We also evaluated 4 granulosa cell tumors (Table 1). Six normal tissues adjacent to tumors and normal ovaries were used as controls. Slides from each case were reviewed for grade following the criteria of the American Joint Committee on Cancer, 1988 edition. HEK293 cells were cultured at 37°C in DMEM supplemented with 10% FCS. Transfection was carried out with calcium phosphate. The relationship between the alteration of BTAK/Aurora-A and tumor grade and stage was analyzed with \( \chi^2 \) tests.

**Expression Constructs, GST Fusion Protein, and Production of Anti-BTAK/Aurora-A Antibody.** The pcDNA3-BTAK/Aurora-A was kindly provided by Dr. Subrata Sen (The University of Texas M. D. Anderson Cancer Center). We subcloned HA epitope-tagged, wild-type BTAK/Aurora-A (1.2 kb) at the Not sites of the mammalian expression vector pHM6 (Boehringer Mannheim). The GST-BTAK/Aurora-A was created by PCR amplification of aurora box-2 of BTAK/Aurora-A (2) using primers 5'-CAGGCTCAGCGGGTCTTGTGTC-3' and 5'-CAGTTCTCTTCAGGATT-3'. The PCR products were inserted into pGEX-4T vector. Logarithmically growing cultures of *Escherichia coli* TOP10 transformed with the pGEX-4T recombinant was incubated with 0.1 mM isopropyl-\( \beta \)-thiogalactopyranoside at 37°C for 6 h. The cells were pelleted, resuspended in cold PBS, and sonicated on ice. Debris was removed by centrifugation, and the supernatant was applied to a glutathione-Sepharose 4B column (Pharmacia Biotech). GST-BTAK/Aurora-A fusion protein was eluted. Anti-BTAK/Aurora-A antibodies were raised in New Zealand White rabbits. Approximately 300 \( \mu \)g of GST fusion protein were used to immunize each rabbit every 2 weeks; rabbits were bled 12 days after each booster injection. The antibodies were affinity purified.

**Immunoprecipitation and Western Blotting Analyses.** The frozen tissue was lysed by a Tissue Tearor in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1% NP40, 5 mM EGTA (pH 7.5), 1 mM EDTA (pH 8.0), 2 mM phenylmethylsulfonyl fluoride, 2 \( \mu \)g/ml aprotinin and leupeptin, 2 mM benzamidine, 10 mM NaF, 10 mM NaP i, 1 mM sodium vanadate, and 25 mM \( \beta \)-glycerolphosphate. Lysates were centrifuged at 12,000 \( \times \) g for 15 min at 4°C. Equal amounts of protein lysate were analyzed for BTAK/Aurora-A expression and enzyme activity. For immunoprecipitation, lysates were precleared with protein A-protein G agarose beads for 4°C for 20 min. After removal of the beads by centrifugation, lysates were incubated with anti-BTAK/Aurora-A polyclonal antibody in the presence of 30 \( \mu \)l of protein A:protein G (2:1) agarose beads at 4°C for 20 min. The beads were washed three times with the lysis buffer. Protein expression was determined by Western blotting analyses probed with anti-BTAK/Aurora-A or anti-HA antibody. Detection of antigen bound antibody was carried out with the ECL Western Blotting Analysis System (Amersham).

**In Vitro Protein Kinase Assay.** The immunoprecipitation for BTAK/Aurora-A kinase assay was performed as described above. The beads were washed three times with lysis buffer and two times with kinase buffer [100 mM Tris-HCl (pH 7.5), 2 mM EDTA (pH 8), 20 mM MgCl 2,10 mM MnCl 2, and 1 mM DTT] in the presence of the protease inhibitors. The reaction was carried out with 10 \( \mu \)Ci of [\( ^{32} \)P]ATP and 3 \( \mu \)M unlabelled ATP in 30 \( \mu \)l of kinase buffer. MBP (4 \( \mu \)g) was used as exogenous substrate. After incubation at 37°C for 30 min, the reaction was stopped by adding protein-loading buffer and separated by SDS-PAGE. Each experiment was repeated three times. The relative amounts of incorporated radioactivity were determined by autoradiography and quantified with a Phosphoimager (Molecular Dynamics).

**Southern and Northern Blotting Analyses.** Genomic DNA and total RNA were isolated from the human ovarian tumor specimens by standard methods (2). Southern blots were prepared by digestion of 10 \( \mu \)g of DNA with EcoRI and detected with a random primer [\( ^{32} \)P]dCTP-labeled BTAK/Aurora-A cDNA probe. Autoradiographs were quantitated relative to \( \beta \)-actin using Image-Quant software. For Northern blotting analyses, 20 \( \mu \)g of total RNA were electrophoresed on agarose gel, transferred, and detected with BTAK/Aurora-A cDNA probe.

**Immunohistochemistry.** Formalin-fixed, paraffin-embedded sections were subjected to antigen retrieval by boiling in 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven after

**Table 1** Alterations of BTAK/Aurora-A and tumor histopathology

<table>
<thead>
<tr>
<th>Histology</th>
<th>Low/no</th>
<th>High/moderate</th>
<th>BTAK kinase activity</th>
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<tbody>
<tr>
<td>LMP</td>
<td>10</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Serous cystadenocarcinoma</td>
<td>58</td>
<td>27</td>
<td>31</td>
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<td>12</td>
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<td>Endometrioid cystadenocarcinocarcin</td>
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<td>Clear cell cystadenocarcinocarcin</td>
<td>1</td>
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<td>0</td>
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<tr>
<td>Granulosa cell tumor</td>
<td>4</td>
<td>4</td>
<td>0</td>
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<tr>
<td>Mixed tumor</td>
<td>1</td>
<td>0</td>
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dewaxing and rehydration. The Vectastain ABC Kit for rabbit IgG (Vector Laboratories) was used to immunostain the tissue sections with anti-BTAK/Aurora-A antibody. Endogenous peroxidase and biotin were blocked, and sections were incubated 1 h at room temperature with a 1:250 dilution of antibody to BTAK/Aurora-A. The remainder of the staining procedure was performed according to the manufacturer’s instructions using diaminobenzidine tetrahydrochloride as the chromogen and hematoxylin for counterstaining. Primary antibody was replaced with an equal concentration of nonimmune rabbit IgG on control sections.

RESULTS
Characterization of BTAK/Aurora-A Antibody. Previous studies have demonstrated amplification of the BTAK/Aurora-A in 10–25% of human ovarian cancer (3, 15). However, alterations of BTAK/Aurora-A at kinase and protein levels have not been documented. To examine BTAK/Aurora-A kinase activity and protein expression in human ovarian carcinoma, we generated rabbit polyclonal anti-BTAK/Aurora-A antibody. The specificity of the antibody was examined with HEK293 cells transfected with the HA-BTAK/Aurora-A expression plasmid. Western blotting analyses revealed that anti-BTAK/Aurora-A antibody reacted strongly with BTAK/Aurora-A protein (Fig. 1A). After preincubation of anti-BTAK/Aurora-A antibody with GST-BTAK/Aurora-A antigen, no BTAK protein was detected (Fig. 1B). In addition, we have further examined the usefulness of the anti-BTAK/Aurora-A antibody for immunoprecipitation. Immunoprecipitates were prepared with anti-BTAK/Aurora-A antibody in HEK293 cells transfected with the HA-BTAK/Aurora-A expression construct, separated in SDS-PAGE, and detected with anti-HA antibody. As shown in Fig. 1C, anti-BTAK/Aurora-A antibody is capable of precipitating BTAK/Aurora-A protein from the cell lysate. These results indicate that our anti-BTAK/Aurora-A antibody specifically reacts with BTAK/Aurora-A and works for both Western blot and immunoprecipitation.

Frequent Activation of BTAK/Aurora-A Kinase in Primary Ovarian Tumors. As BTAK/Aurora-A is a serine/threonine kinase and plays a significant role in cell proliferation by phosphorylation of downstream targets, such as histone H3 and CPEB (7–9), we have examined BTAK/Aurora-A kinase activity in human primary ovarian carcinoma. In vitro BTAK/Aurora-A kinase assays were performed in 92 specimens using MBP as substrate. Fig. 2A shows that the in vitro kinase conditions that we used could detect high levels of BTAK/Aurora-A kinase activity in BTAK/Aurora-A- but not pcDNA3-transfected HEK293 cells. Activation of BTAK/Aurora-A, defined as an average reading of the kinase activity 4-fold higher than that in normal ovarian tissues, was detected in 44 (48%) ovarian cancer specimens. The results were confirmed by triplicate experiments. Fig. 2B represents typical examples of BTAK/Aurora-A activation in ovarian tumors.

In addition, we have examined the relationship between kinase activity and protein level of BTAK/Aurora-A. Western blot and immunohistochemical staining analyses revealed that all of the 44 cases with elevated levels of BTAK/Aurora-A kinase overexpressed BTAK/Aurora-A, indicating that activation of BTAK/Aurora-A is largely attributable to increased expression levels of BTAK/Aurora-A protein. To determine whether elevated protein levels of BTAK/Aurora-A result from its alterations at DNA and/or mRNA levels, we performed Northern and Southern blotting analyses in 43 tumor specimens and 6 normal ovarian tissues. Overexpression of BTAK/Aurora-A mRNA was detected in 18 (42%) tumors, in which BTAK/Aurora-A protein was elevated, whereas amplification of the BTAK/Aurora-A (>3-fold higher than that in normal ovary) was only observed in 6 (14%) specimens, all of which also exhibited overexpression of the BTAK/Aurora-A mRNA and protein. Representative examples are shown in Fig. 3, B and C, suggesting that BTAK/Aurora-A could be regulated at transcription, translation, and/or post-translational levels.

Elevated Expression of BTAK/Aurora-A Protein in Human Ovarian Carcinomas. We next examined the expression of BTAK/Aurora-A protein in the same series of human ovarian tumors. Western blotting analyses revealed high levels of BTAK/Aurora-A protein in 52 of 92 (57%) tumor specimens (Fig. 3A and Table 1). To confirm this result, we have carried out immunohistochemical staining of paraffin sections with anti-BTAK/Aurora-A.
antibody. A moderate to strong predominantly cytoplasmic BTAK/Aurora-A expression was detected in the same 52 ovarian tumors (Fig. 4) that overexpress BTAK/Aurora-A protein revealed by Western blotting analyses. It has been shown that although BTAK/Aurora-A protein localizes to both the cytoplasm and nucleus, it is mainly in cytoplasm (16). Strong immunoreaction of BTAK/Aurora-A was observed in tumor cells but not normal ovarian epithelium. There was no preferential BTAK/Aurora-A expression among the three major histopathological types of ovarian surface epithelial carcinomas (serous, mucinous, and endometroid). Four
ovarian granulosa cell tumors exhibited no detectable BTAK/Aurora-A, implying that the expression of BTAK/Aurora-A could be restricted to ovarian epithelial neoplasm. Notably, activation/overexpression of BTAK/Aurora-A is more frequently detected in LMP (8 of 10) than serous and mucinous cystadenocarcinoma (39 of 70). Furthermore, we have observed that BTAK/Aurora-A is preferentially expressed in low-grade [25 of 39 (65%) grade I/II versus 13 of 33 (40%) grade III] and early stage tumors [24 of 35 (67%) stage I/II versus 9 of 20 (45%) stage III/IV] (Tables 2 and 3). However, there is no preference in six cases with amplification of BTAK/Aurora-A (one case is stage I, two cases are stage II, and the rest are stage III). In addition, invasive tumors exhibit much less BTAK/Aurora-A immunoreactivity compared with the noninvasive tumors (Fig. 4). Interestingly, even in the same tumor, BTAK/Aurora-A immunoreactivity was seen to pale at the invasive front of the tumor, whereas the noninvasive portion of the tumor stained strongly. Fig. 4D shows an invasive serous adenocarcinoma exhibiting BTAK/Aurora-A expression. However, the intensity of the staining was much less in the infiltrating component compared with the LMP component lining the surface. These data suggest that alterations of BTAK/Aurora-A could be an early event in the development of human ovarian cancer, although there was no statistic significance at the kinase level between early stage/low-grade and late stage/high-grade tumors (Tables 2 and 3).

**DISCUSSION**

Ovarian cancer is thought to arise from alterations in genes involved in regulating cell proliferation, apoptosis, and genomic integrity. Alterations in several proto-oncogenes and tumor sup-

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*Fig. 4* High levels of BTAK/Aurora-A protein expression in low-grade and less invasive tumors. Immunohistochemical staining of the paraffin sections prepared from mucinous papillary tumors of low malignant potential (*A* and *B*), invasive mucinous adenocarcinoma (*C*), and serous adenocarcinoma (*D–F*) with anti-BTAK/Aurora-A antibody. The intensity of the immunostaining in invasive tumors is much weaker or absent in comparison with noninvasive tumors.
pressor genes have been described. The ERBB2, PIK3CA, and AKT2 oncogenes are frequently amplified and subsequently overexpressed in ovarian cancers (17–19). Overexpression of ERBB2 or AKT2 correlates with poor prognosis of the patients (17, 18). Amplification of the MYC oncogene has been detected in ~20% of ovarian cancers, more frequently in serous than in mucinous cancers (20). Other oncogenes altered in ovarian cancer include KRAS, INT2, FMS, and MDM2, but these alterations appear to be relatively uncommon (17). Cytogenetic and comparative genome hybridization studies have revealed frequent gains in chromosome 20q11–20q13.2 amplicon (3, 15, 22). Amplification of the BTAK/Aurora-A gene at 20q11, AIB1 gene at 20q12, MYBL2 and phosphotyrosine-phosphatase 1 genes at 20q13, and ZNF217 and BTAK/Aurora-A genes at 20q13.2 amplicon (3, 15, 22). Amplification of the BTAK/Aurora-A has been reported in 15–25% ovarian cancer cell lines and primary tumors (3, 15). In the present study, we have studied kinase activity and protein expression of BTAK/Aurora-A in primary ovarian carcinomas. Elevated kinase activity and protein levels of BTAK/Aurora-A were detected in 48 and 57% of the tumors examined, respectively. These data indicate that alterations of BTAK/Aurora-A at kinase and protein levels are frequent changes in human ovarian cancer. Thus, BTAK/Aurora-A could play a pivotal role in the pathogenesis in the majority of cases of this malignancy.

We have also examined DNA amplification and mRNA overexpression of the BTAK/Aurora-A in 43 tumors. Frequency of alterations of the BTAK/Aurora-A at DNA level is much lower than at protein and kinase levels. Moreover, overexpression of BTAK/Aurora-A mRNA is much more common than amplification of BTAK/Aurora-A. A possible reason for these findings is insensitive detection of amplification by Southern blot analyses. Interphase fluorescent in situ hybridization has been reported to be more sensitive for detection of gene amplification. A recent report using fluorescent in situ hybridization demonstrated amplification of BTAK/Aurora-A in 6 of 24 (25%) sporadic ovarian carcinomas (15). A second possibility to explain the findings relates to the transcriptional regulation of BTAK/Aurora-A. In addition, our data show that overexpression of BTAK/Aurora-A protein is ~20% higher than overexpression of its mRNA in the human primary ovarian tumors examined, which could be because of RNA quality. However, based on 28S and 18S bands in our Northern blots, RNA degradation did not occur in any of the 43 tumors we examined (Fig. 3), suggesting that translational and/or post-translational regulation could be involved in up-regulation of BTAK/Aurora-A protein in human ovarian carcinoma.

We have also observed that BTAK/Aurora-A kinase activity correlates well with its protein expression in ovarian tumor specimens examined, except in 8 cases exhibiting increased protein but not kinase levels of BTAK/Aurora-A. We and others (23, 24) have shown previously that Akt protein kinase and Stat3 DNA-binding activities in primary tumors are closely associated with the interval time to freezing the specimen after surgical resection. The 8 ovarian tumors, which have elevated levels of BTAK/Aurora-A protein but not BTAK/Aurora-A kinase, also overexpress AKT2 protein but have low levels of AKT2 kinase activity (18), indicating that these results could be attributable to improper processing and/or storage of the specimens. Nevertheless, the majority of tumors with overexpressed BTAK/Aurora-A protein displayed increased levels of BTAK/Aurora-A kinase activity, suggesting that the activation of BTAK/Aurora-A is largely caused by overexpression of its protein in the primary ovarian tumors examined in this study.

The relationship between overexpression of BTAK/Aurora-A and tumor grade/stage is controversial. A previous study in ductal breast cancer showed that overexpression of BTAK/Aurora-A protein was independent of tumor histopathological type and lacked correlation with tumor size and lymph node metastases (16). Other studies showed that alterations of BTAK/Aurora-A associate with poor prognosis in gastric cancers and high grade/late stage in breast and bladder cancer (25–27). In the present study, however, we observed that BTAK/Aurora-A protein kinase is preferentially activated/overexpressed in low-grade and early stage ovarian cancer, as well as LMP (Tables 1–3), although there was no statistic significance at the kinase level between low-grade/early stage and high-grade/late stage tumors. Moreover, immunohistochemical staining showed that BTAK/Aurora-A is preferentially expressed in less invasive tumors and declines once a tumor becomes invasive (Fig. 4, D–F). A recent report using a well-established rat mammary cancer model demonstrated that amplification of the BTAK/Aurora-A is an early genetic change during the development of
Activating BTAK/Aurora-A Kinase in Human Ovarian Cancer


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