Overexpression of Aurora-A Enhances Invasion and Matrix Metalloproteinase-2 Expression in Esophageal Squamous Cell Carcinoma Cells

Xiaoxia Wang, Na Lu, Bo Niu, Xianjiu Chen, Jun Xie, and Niuliang Cheng

Abstract
Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive cancers, and metastasis is the principal cause of death in ESCC patients. It has been shown that amplification and overexpression of mitotic serine/threonine kinase Aurora-A occur in several types of human tumors, including ESCC. Moreover, increase in expression levels of Aurora-A has been predicted to correlate with the grades of tumor differentiation and invasive capability. However, the mechanisms by which Aurora-A mediates its invasive effects still remain elusive. In this article, we showed that Aurora-A overexpression significantly increased cell migration and invasion as well as secretion and expression of matrix metalloproteinase-2 (MMP-2). Conversely, siRNA-mediated knockdown of Aurora-A expression in human ESCC cells led to inhibition of cell invasiveness as well as secretion and expression of MMP-2. In addition, Aurora-A overexpression increased phosphorylation levels of p38 mitogen-activated protein kinase (MAPK) and Akt, and the knockdown of Aurora-A by siRNA decreased the activity of p38 MAPK and Akt. Moreover, the blocking of the activity of above kinases using chemical inhibitors suppressed the ability of Aurora-A to induce MMP-2 secretion and expression as well as cell invasion. These data show that overexpression of Aurora-A contributes to the malignancy development of ESCC by enhancing tumor cell invasion as well as MMP-2 activity and expression, which can occur through signaling pathways involving p38 MAPK and Akt protein kinases. Taken together, these studies provide a molecular basis for promoting the role of Aurora-A in malignancy development of ESCC.

Introduction
Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive carcinomas of the gastrointestinal tract. Despite improvements in detection, surgical resection, and adjuvant therapy, the overall prognosis for esophageal cancer patients remains poor. The very low overall survival for ESCC may in part be attributed to the high invasive potential and distant metastasis (1). Therefore, better understanding of the molecular mechanisms of invasion and metastasis of ESCC will aid in the development of new strategies for curative treatment of ESCC.

Aurora-A, a member of the serine/threonine kinase family of proteins, is a centrosome-associated protein and has been implicated in regulating centrosome function, spindle assembly, chromosome segregation, and cytokinesis (2). In recent years, increased attention has now been focused on Aurora-A kinase because of its suggestive role in tumorigenesis. Numerous studies have shown that amplification and overexpression of Aurora-A occur in several types of human tumors, including ovarian cancer (3), breast cancer (4), gastric cancers (5), ESCC (6, 7), and so on. Furthermore, increase in expression levels of Aurora-A has been predicted to correlate with the grades of tumor differentiation and invasive capability (6, 8). The molecular mechanism underlying the induction of invasion by the Aurora-A, however, has not been defined yet. Therefore, elucidation of the molecular mechanism for the promotion of ESCC invasion by Aurora-A may lead to the development of new approaches for effective therapy.

Cancer cell invasion and metastasis is a complex, multistep process, of which proteolytic degradation of extracellular matrix (ECM) exerted by matrix metalloproteinases (MMP) is considered to be an essential step (9, 10). Among this group of endopeptidases, MMP-2 specifically degrades type IV collagen, the main component of the basement membrane, and seems to play a crucial role in tumor invasion and metastasis (11). MMP-2 is known to be produced as a pro-MMP-2 (72 kDa) and to be an active-MMP-2 (62 kDa) by proteolytic processing. Increases in activity and expression of MMP-2 have been frequently observed in many human cancers with invasive and metastatic capability. Previous studies have shown that expression of MMP-2 in ESCC is significantly associated with the tumor invasion depth and
lymph node metastasis (12–14). However, the regulatory mechanism of MMP-2 expression has not been well characterized.

Because Aurora-A is overexpressed in most of tumors and its role in invasion and metastasis has been reported, we hypothesize that some of the functions of Aurora-A may be mediated through the regulation of expression and activity of MMP-2 metalloproteinase. In this study, we show that overexpression of Aurora-A promotes invasion as well as secretion and expression of MMP-2 in ESCC cells. Conversely, siRNA-mediated knockdown of Aurora-A in ESCC cell lines results in a decrease in invasion as well as activity and expression of MMP-2. In addition, by using chemical inhibitors, we show that Aurora-A upregulates MMP-2 and promotes invasion through pathways dependent on p38 mitogen-activated protein kinase (MAPK) and Akt signaling. Collectively, these findings provide new evidence that the overexpression of Aurora-A contributes to the malignancy development of ESCC.

Materials and Methods
Cell culture and reagent
Human ESCC KYSE150 (15) and EC9706 (16) cells were from the Tumor Cell Bank of Chinese Academy of Medical Sciences and cultured in RPMI 1640 medium (Gibco-BRL) supplemented with 10% FBS (HyClone), 100 U/mL of penicillin, and 100 μg/mL streptomycin at 37°C, and 5% CO₂. Polyclonal antibodies for Aurora-A, phospho-extracellular signal-regulated kinase (ERK)1/2 (Thr202/Tyr204), ERK1/2, phospho-38 MAPK (Thr180/Tyr182), p38 MAPK, phospho-Akt (Ser473), and Akt were purchased from Cell Signaling Technology Company. MMP-2 polyclonal antibody and anti-β-actin monoclonal antibody were purchased from Santa Cruz Biotechnology. The chemical inhibitors, SB203580, LY294002, PD98059, and the MMP inhibitor, GM6001 were purchased from Santa Cruz Biotechnology. Aurora-A, AUG CCC UGU CUU ACU GUC A, according to the siRNA sequence that we used is listed as the following: Aurora-A, UUC UCC GAA CGU GUC ACG U. Cells were transfected with Aurora-A siRNA or control siRNA using Lipofectamine 2000 for 48 hours, after which they were fixed, stained, photographed, and counted.

Expression vector and transfection
The open reading frame for Aurora-A was cloned into mammalian expression vectors (pEGFP-C1) by standard PCR cloning methods. Aurora-A expression construct was transfected into cells using Lipofectamine 2000 (Invitrogen). Transfected cells were selected by 0.4 mg/mL G418 sulfate. Stable transfectant clones were characterized by Western blot for their expression levels of Aurora-A protein.

Western blot analysis
Proteins in cell lysates were prepared using a previously described method (6), separated by SDS-PAGE and electrotransferred to nitrocellulose membrane, which were subsequently blotted using the indicated antibodies and visualized by the enhanced chemiluminescence detection system (Pierce). The band intensities were analyzed by densitometry using Kodak Molecular Imaging Software.

Wound-healing experiment
Cells were grown in 35-mm culture dishes to 90% confluency. A wound was formed using a 200-μL pipette tip to clear the cell monolayer. After washing with PBS, serum-free medium was added. Photographs of the wounded area were taken immediately after making the scratch (0-hour time point) and after 24 hours to monitor the invasion of cells into the wounded area. Quantification was done by measuring the distances covered by the cells (wound width) that were plotted in term of pixels.

Invasion assay
The invasive properties of cells were assayed in a modified Boyden chamber with a membrane filter. In brief, cells were trypsinized with trypsin-EDTA and plated in the upper chambers in serum-free RPMI 1640 medium with or without GM6001 or chemical inhibitors (1 × 10⁵ cells per mL at 50 μL per well). The serum-free RPMI 1640 medium was placed in the lower compartment (30 μL per well). The 2 compartments were separated by 1.5% gelatin-coated polycarbonate filters with 8-μm pores (Neuro Probe). The system was incubated for 48 hours (37°C, 5% CO₂). Cells from the upper side of the membrane were removed by wiping with cotton. Then membranes were fixed, stained, photographed, and counted.

siRNA knockdown experiments
Aurora-A siRNA was synthesized by Genechem. The siRNA sequence that we used is listed as the following: Aurora-A, AUG CCC UGU CUU ACU ACU GUC A, according to positions 853–871 within the Aurora-A mRNA; control siRNA, UUC UCC GAA CGU GUC ACAG U. Cells were transfected with Aurora-A siRNA or control siRNA using Lipofectamine 2000 for 48 hours, after which they were harvested for Western blot analysis and reverse transcriptase PCR or used for invasion assays. In addition, culture media collected from the siRNA-transfected cells were analyzed for MMP-2 activity as described below.

Gelatin zymography
Equal amounts of proteins from serum-free media conditioned for 48 hours were mixed with an equal volume of 2 × SDS sample buffer and loaded without boiling onto 10% SDS–PAGE gel containing 0.1% gelatin. After electrophoresis, the gels were then incubated for 30 minutes at room temperature in 2.5% Triton X-100 twice, rinsed and incubated for 48 hours at 37°C in substrate buffer [50 mmol/L Tris-HCl (pH 7.5), 50 mmol/L NaCl, 10 mmol/L CaCl₂]. Following incubation, the gel was stained with 0.05% Coomassie brilliant blue G-250 and destained in 10% acetic acid and 20% methanol. Gelatinolytic activity was detected as transparent bands on a blue background, and the band intensities were analyzed by densitometry using Kodak Molecular Imaging Software.

Semiquantitative RT-PCR analysis
Total cellular RNA was purified from the cultured cells using TRIzol reagent (Invitrogen) according to the
manufacturer’s protocol. First strand cDNA synthesis was carried out with 0.5 μg of total RNA using a cDNA synthesis kit (Takara Bio Inc.). The conditions of PCR for MMP-2 and glyceraldehyde phosphate dehydrogenase (GAPDH) were 95°C (30 seconds), 60°C (30 seconds), and 72°C (60 seconds) for 30 cycles. The primers were 5’-CAC TTT CCG GAA CAA AT-3’ (forward), 5’-CTC AGC CTT TGC TGT-3’ (reverse) for MMP-2; 5’-GGT CCG GAG AAC GGG AAG CTT G-3’ (forward), 5’-GAG GCT GGT GCT AAG CAG TT-3’ (reverse) for GAPDH.

A5 -mL volume of each reaction was then loaded onto 1.5% agarose gels, stained with ethidium bromide, and the band intensities were analyzed by densitometry using Kodak Molecular Imaging Software.

Chemical inhibition

An ERK inhibitor (PD98059), an Akt inhibitor (LY294002), and a p38 MAPK inhibitor (SB203580) were used in our assays to determine the signaling pathways involved. Cells were cultured to around 80% confluence and pretreated with or without inhibitors in serum-free medium for 48 hours.

Statistical analysis

All data represented at least 3 independent experiments and were statistically evaluated by Student t test with SPSS 11.5 statistic software. The results were expressed as mean ± SD. P < 0.05 was considered statistically significant.

Results

Generation of Aurora-A–transfectant clones

To study the possible roles of Aurora-A protein in ESCC cells, human ESCC cell line KYSE150 was transfected with either a control empty vector or an Aurora-A cDNA expression vector. Western blot analysis showed that the selected stable clones (named as Aur-1 and Aur-2) expressed fusion protein of GFP–Aurora-A (Fig. 1A). Compared with the relatively low levels of endogenous Aurora-A in control clone generated with an empty vector (named as Con), Aur-1 and Aur-2 clones expressed at least 2- to 3-fold more Aurora-A protein (Fig. 1B).

Effect of Aurora-A overexpression on ESCC cell migration and invasion in vitro

To determine the effect of Aurora-A overexpression on cell migration, we carried out wound healing assays in 35-mm culture plates. After 24 hours approximately, Aur-1 and Aur-2 cells efficiently spread into the wound area to such an extent that the wound boundary was almost indistinguishable, whereas a very few cells in control group spread forward (Fig. 1C). Cell migration was quantified by measuring wound widths that were plotted...
in term of pixels. As shown in Fig. 1D, cellular migrations were increased by up to 90% at 24 hours in Aur-1 and Aur-2 cells compared with control cells. These results suggested that Aurora-A overexpression may promote cell migration.

To further investigate whether Aurora-A overexpression can modify tumor cell invasion, we examined the invasion activity by an in vitro Boyden chamber invasion assay. After 48 hours, consistent with the wound-healing assay results, tumor cell invasion through gelatin (a denatured form of collagen) was significantly higher in Aur-1 and Aur-2 cells compared with control cells. The analysis of quantification showed that invading cells of Aur-1 and Aur-2 had an average 25-fold increase compared with control cells (Fig. 1E). To further confirm the role of Aurora-A overexpression on invasive properties of ESCC cells, we carried out siRNA-mediated knockdown of Aurora-A expression in ESCC cell line EC9706, which caused 4- to 5-fold reduction of Aurora-A expression compared with control siRNA-treated cells (Fig. 2A and B). The results in Fig. 3C illustrated that inhibition of Aurora-A expression in EC9706 cells significantly reduced the invasive potential (3- to 4-fold) of these cells. These data showed a critical role for Aurora-A overexpression in the invasion of ESCC cell line.

MMPs are known to be crucial for degrading extracellular matrix components and for promoting tumor invasion. To further show that Aurora-A overexpression increased ESCC cell invasion was due to the induction of MMPs, we used GM6001, a chemically synthesized MMP inhibitor in the invasion experiments. We found that Aurora-A overexpression–induced ESCC cell invasion was significantly inhibited by GM6001 (Fig. 1E). Together, these results suggested that increase of cell invasive capacity by overexpression of Aurora-A is MMP dependent.

Modulation of the activity and expression levels of MMP-2 by Aurora-A overexpression

As inhibition of MMPs reversed Aurora-A overexpressing induced cancer cell invasion through gelatin, we next studied whether overexpression of Aurora-A increase activity of MMP-2. Zymographic analysis showed that the active form of MMP-2 was elevated in Aur-1 and Aur-2 cells when compared with control cells (4- to 5-fold; Fig. 3A, left). Furthermore, because tumor invasion and metastasis are often associated with enhanced synthesis of MMP-2, we examined the effect of overexpression of Aurora-A. We first investigated the effect of overexpression of Aurora-A on MMP-2 mRNA levels by RT-PCR. MMP-2 mRNA levels were increased by 5- to 6-fold in Aur-1 and Aur-2 cells compared with control cells (Fig. 3A, middle). Then we further investigated whether the elevated mRNA levels of MMP-2 were also correlated with protein expression levels. Consistent with the RT-PCR results, overexpression of Aurora-A enhanced MMP-2 protein expression (2- to 3-fold; Fig. 3A, right). These data suggested a correlation between Aurora-A expression and MMP-2 expression and secretion. Furthermore, to verify the specific requirement for Aurora-A expression in MMP-2 expression and secretion, EC9706 cells were transfected with siRNA specific for Aurora-A. The knockdown of Aurora-A caused 3- to 5-fold reduction of MMP-2 secretion and expression (Fig. 3B). Therefore, these results suggested that overexpression of Aurora-A can upregulate activity and expression of MMP-2.

Signaling pathways for the Aurora-A–stimulated MMP-2 upregulation

To elucidate the signaling pathway(s) involved in the upregulation of MMP-2 by Aurora-A, we examined the activities of some signaling molecules. The phosphorylation levels of both p38 MAPK and Akt in Aur-1 and Aur-2 cells...
were much higher than control cells, whereas the total amounts remained unchanged. Conversely, silencing of Aurora-A resulted in a significant decrease in the amounts of phosphorylated forms of both p38 MAPK and Akt kinases, whereas the total amounts remained unchanged. However, little significant differences in the phosphorylation levels of ERK1/2 were observed between the Aur-1, Aur-2, or Aurora-A siRNA transfected cells and control cells (Fig. 4).

These results suggested that the signaling pathways composed of p38 MAPK and Akt may participate in the Aurora-A–induced upregulation of MMP-2. We next examined the activity and expression levels of MMP-2 as well as invasion in the Aurora-A–transfected cells in the presence of inhibitors of p38 MAPK, Akt, and ERK1/2 for the 48 hours culture period. As expected from above results, in gelatin zymography, the activities of MMP-2 in the Aur-1 and Aur-2 cells were significantly attenuated by SB203580 (a p38 MAPK inhibitor) and LY294002 (an Akt inhibitor) in a dose-dependent manner with statistical significance achieved at 10 μmol/L, the lowest dose tested. However, the inhibitor PD98059 (an ERK inhibitor) exerted little inhibition effect on the Aurora-A–stimulated MMP-2 activity (Fig. 5). Consistent with the gelatin zymography results, mRNA and protein expression levels of MMP-2 in the Aur-1 and Aur-2 cells were significantly attenuated by SB203580 and LY294002 in a dose-dependent manner.
with statistical significance achieved at 10 to 20 μmol/L. PD98059 did not affect the Aurora-A–induced MMP-2 upregulation (Figs. 6A and B). In addition, invasion abilities of Aur-1 and Aur-2 cells were also significantly inhibited by SB203580 or LY294002 in a dose-dependent manner with statistical significance achieved at 10 to 20 μmol/L, whereas PD98059 did not affect invasive phenotypes (Fig. 7). Furthermore, these inhibitors had no effects on cell proliferation/survival, excluding nonspecific toxicity of the agent at these concentrations (data not shown). These data showed that the Aurora-A–induced upregulation of MMP-2 and invasive phenotypic change require activation of p38 MAPK and Akt pathways, but not that of ERKs signaling.

**Discussion**

Aurora-A is a potent oncogene. It is expressed at high levels in several types of human tumors. Its oncogenic potential has been shown by its overexpression in murine fibroblasts as well as mammary epithelia, which show centrosome amplification, aneuploidy, and oncogenic phenotype (4, 17). High levels of Aurora-A expression have been reported to correlate with parameters of clinical aggressiveness including high histologic grade, invasion, and increasing rate of metastasis (6–8). Recent studies also indicate that Aurora-A can enhance cell migration, and inhibition of Aurora-A can suppress cell invasion in vitro (18–21). In addition, previous studies have shown that Aurora-A protein is able to physically associate with multiple important cellular proteins such as p53, BRCA1, NF-κB, Plk1, and mTOR (22–26), to disrupt/alter their physiologic functions. These studies indicate that cell signaling cross-talk between Aurora-A and other cellular proteins are likely essential for tumor occurrence and malignant development. However, the mechanisms by which Aurora-A mediates its invasive and metastatic effects still remain elusive. Several studies have reported that MMP-2 is upregulated in many types of human cancer, and its expression levels are often associated with cell invasion and metastasis (9, 14).

On the basis of this information, we hypothesize that there exists a relationship between Aurora-A and MMP-2, and some of the oncogenic functions of Aurora-A may be mediated through the modulation of expression and activity of MMP-2. Therefore, in this study, we first sought to identify the role of Aurora-A in the regulation of invasive tumor cells and found that migration and invasion of ESCC cells in vitro could be elevated by overexpression of Aurora-A. Furthermore, our results showed for the first time that...
overexpression of Aurora-A increased activity and expression of MMP-2. Addition of GM6001, the broad spectrum MMP inhibitor, inhibited cell invasion, suggesting that Aurora-A enhanced ESCC cell invasiveness at least partly mediated by increased MMP-2 activity and expression. Consistent with above hypothesis, siRNA-mediated knockdown of Aurora-A in ESCC cell lines resulted in a decrease in invasion as well as activity and expression of MMP-2.

Figure 6. Effects of signaling molecule inhibitors on the Aurora-A–induced MMP-2 expression in the Aurora-A–transfectant clones. The Aur-1 and Aur-2 cell were cultured in serum-free medium with different concentrations of SB203580, LY294002, or PD98059. Following 48 hours of incubation, the cells were collected and analyzed with (A) RT-PCR and (B) Western blot using anti–MMP-2 antibody. GAPDH and β-actin were used as the internal control. The graph represents densitometry of the results of 3 independent experiments (mean ± SD). *, Statistically different, compared with the nontreated Aurora-A–transfectant cells (P < 0.05).
Together, our data illustrate the crucial role of Aurora-A in the regulation of MMP-2 activity and expression as well as invasion of ESCC using in vitro cell models. In addition, to further provide evidence of Aurora-A contributing to the malignancy development of ESCC and refine potential therapeutic strategies targeted against ESCC invasion, the role of Aurora-A in regulation of tumor cell invasion and MMP-2 in vivo is currently underway.

Metastasis is a complex process of tumor cell invasion to new tissues. Tumor-associated MMPs are important components in the metastatic process through their capacity to degrade extracellular matrix proteins. MMP-2 is one of the most vital enzymes for the degradation of the main constituent of the basement membrane and therefore deeply involved in cancer invasion and metastasis (11). Thus, elucidation of MMP-2 activation and expression mechanism will help understand the process of cell invasion and metastasis. The MAPK family, the ERK, the c-jun-NH₂-kinases, and the p38 MAPK kinase are activated via reversible phosphorylation and mediate signal that regulate cell growth and differentiation, gene expression, protein synthesis, and secretion (27). In addition, phosphoinositide 3-kinase–Akt signaling plays a prominent role in several processes considered the hallmark of cancer (28). Furthermore, MAPK and Akt pathways have been shown to play a role in the regulation of MMP secretion and expression (29). Therefore, we hypothesized that Aurora-A upregulated MMP-2 by interacting with the ERK1/2, p38 MAPK, or Akt pathways detected phosphorylation levels of these proteins, which were assumed to reflect the activation of the proteins. The higher phosphorylation levels of p38 MAPK and Akt were observed in the Aurora-A–transfectant cells than those in the mock-transfectant cells. On the contrary, silencing of endogenous Aurora-A kinase with siRNA decreased p38 MAPK and Akt activation. The participation of p38 MAPK and Akt in Aurora-A signaling pathway(s) became apparent when some inhibitors specific to signal transducing molecules, SB203580, a p38 MAPK inhibitor, and LY294002, an Akt inhibitor, suppressed the induction of MMP-2 secretion and expression as well as invasion by Aurora-A in ESCC cells. Moreover, the inhibitory effects of SB203580 and LY294002 were not due to nonspecific toxicity because the viability of cells was not modified by SB203580 and LY294002. The fact that the inhibitory effects of SB203580 and LY294002 were observed at concentrations as low as 10 to 20 μmol/L indicates that p38 MAPK and Akt are involved, and virtually excludes nonspecific effects. These results are consistent with previous finding that Aurora-A overexpression can upregulate phosphorylation level of Akt (30). However, we also found here that the Aurora-A–stimulated MMP-2 secretion and expression are not mediated by ERK signaling pathway, although some reports show that Aurora-A can enhance the phosphorylation level of ERK (31). This discrepancy can be attributed to the difference in used cancer types. Therefore, the data in this study show that Aurora-A activates p38 MAPK and Akt signaling pathways, instead of the classical ERK1/2 signaling pathway, leading to increases in MMP-2 activity and expression as well as invasion of the cells in vitro.

In conclusion, our study clearly indicates that Aurora-A increases tumor cell invasive properties as well as MMP-2 mRNA level, protein level, and enzymatic activities which can occur through signaling pathways involving p38 MAPK and Akt protein kinases. These findings provide a novel role of Aurora-A in malignancy development in ESCC, and we conclude that Aurora-A may serve a potential molecular target for cancer treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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