

Overexpression of Aurora-A Contributes to Malignant Development of Human Esophageal Squamous Cell Carcinoma

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ABSTRACT

Purpose: Aurora-A/STK15/BTAK, a centrosome-associated oncogenic protein, is implicated in the control of mitosis. Overexpression of Aurora-A has been shown to result in chromosomal aberration and genomic instability. Multiple lines of evidence indicate that Aurora-A induces cell malignant transformation. In the current study, we are interested in investigating the expression of Aurora-A in human esophageal squamous cell carcinoma (ESCC) and characterizing the association of Aurora-A with ESCC-malignant progression.

Experimental Design: Aurora-A protein expression was examined in 84 ESCC tissues and 81 paired normal adjacent tissues by either immunohistochemistry or Western blot analysis. In addition, a gene-knockdown small interfering RNA technique was used in ESCC cells to investigate whether Aurora-A contributes to the ability of a tumor to grow invasively.

Results: The amount of Aurora-A protein in ESCC was considerably higher than that in normal adjacent tissues. Overexpression of Aurora-A was observed in 57 of 84 (67.5%) ESCC samples. In contrast, <2% of normal adjacent tissue displayed high expression of Aurora-A. Interestingly, overexpression of Aurora-A seemed to correlate with the invasive malignancy of ESCC. Disruption of endogenous Aurora-A using small interfering RNA technique substantially suppressed cell migrating ability.

Conclusion: The findings presented in this report show that Aurora-A expression is elevated in human esophageal squamous cell carcinoma and is possibly associated with tumor invasion, indicating that overexpression of Aurora-A may contribute to ESCC occurrence and progression.

INTRODUCTION

Human esophageal squamous cell carcinoma (ESCC) is one of the most frequent malignancies worldwide and occurs at a very high frequency in the People's Republic of China, South Africa, France, and Italy (1). A number of epidemiological investigations have shown that esophageal carcinogenesis and the malignant development of esophageal cancers are complex and associated with multiple etiologic factors, including genetic backgrounds, environmental stimuli, nutritional conditions, and cultural habits (2). Despite some epidemiological observations, the biological mechanism(s) that is involved in ESCC occurrence and progression remains to be elucidated. It has been shown that point mutations of the tumor suppressor gene p53 are detected in 40% of human esophageal cancers (3–5). The Rb gene is also frequently mutated in ESCC (6, 7). Amplification of the cellular protooncogenes Myc, EGFR, HST1, INT2, and cyclin D1 (8–11) are often found in this malignant disease. Jiang *et al.* (11) has reported previously that amplification of the cyclin D1 gene is observed in about 25% of primary esophageal carcinomas from the People's Republic of China. In addition, altered gene expression of some growth- or differentiation-related genes were found recently to be associated with ESCC (12–14). Therefore, abnormalities in oncogenes and tumor suppressor genes, as well as in cell cycle regulators may contribute to esophageal carcinogenesis and malignancy development.

Aurora-A (also designated as STK15, BTAK, or ARK1), a member of a new serine/threonine kinase family, is a centrosome-associated protein and has been implicated in regulating centrosome function, spindle assembly, spindle maintenance, chromosome segregation, and cytokinesis. Suppression of Aurora-A results in defects in mitotic process, incomplete cytokinesis, and genomic instability (15–20). Aurora-A protein consists of 403 amino acids and is able to physically associate with multiple important cellular proteins such as p53, BRCA1, and TACC1 (21–24). The interactions of Aurora-A with those critical molecules have been shown to disrupt/alter their physiological functions and may play roles in tumorigenesis (25, 26). Expression of Aurora-A can up-regulate telomerase activity and thus promote cell transformation (27). Importantly, amplification and overexpression of Aurora-A have been found in several types of human tumors, including breast cancer (28), hepatocellular cancer (29), bladder cancer (30), testicular germ cell tumors (31), non-Hodgkin's lymphoma (32), and pancreatic cancer (33), although the exact roles of Aurora-A in the development of those tumors are currently under further investigation.

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However, Aurora-A expression has not been characterized previously in human ESCC, although a recent demonstration indicates that Aurora-A polymorphisms are associated with advanced disease status of ESCC (34). In the present study, we have examined the association of Aurora-A protein expression with ESCC in both clinical patient samples and tumor cell lines. Our results provide strong evidence that Aurora-A is overexpressed in human ESCC and may play a role in carcinogenesis and malignancy development of ESCC.

MATERIALS AND METHODS

Clinical Tissue Sample Collections. Fresh tumor tissues and normal adjacent tissues were collected from patients with pathologically and clinically confirmed ESCC. Most of the tumor samples were fixed in formalin, embedded in paraffin, and sectioned at 5 $\mu\text{mol/L}$ thick. One section was stained with H&E for histologic examination, and the others were used for immunohistochemistry or immunofluorescence staining. A portion of tumor specimens were kept in liquid nitrogen and sectioned for protein extraction. The clinical consultation reports were available for all tumor samples and the institutional Review Board approved use of the tumor specimens in this study.

Paraffin-embedded tumor samples and normal adjacent tissues from 64 patients were used to immunohistochemically analyze the correlation of Aurora-A expression to pathologic characteristics of the tumors. In addition, 20 tumor and normal adjacent tissues were used for cellular protein extraction and analysis of Aurora-A expression by Western blotting assay.

Immunohistochemical Analysis of Aurora-A Expression. The sample sections were deparaffinized in xylene and rehydrated in graded ethanol. After antigen retrieval with sodium citrate, sections were blocked with 1.5% normal blocking serum in PBS for 1 hour at room temperature and incubated with anti-Aurora-A antibody (Cell Signaling Technology, Inc., Beverly, MA) at 4°C overnight. Sections were then washed with PBS three times and incubated with poly streptavidin-horseradish peroxidase-antirabbit/antimouse immunoglobulin G complex solution for 30 minutes at room temperature. Finally, sections were reacted with H_2O_2 -diaminobenzidine at room temperature for 2 to 3 minutes or until desired stain intensity developed. All sections were counterstained with hematoxylin, followed by dehydration and mounting slides.

Semiquantitative Evaluation of Immunohistochemical Staining. In immunohistochemical analysis, visible brown granules in the cytoplasm were determined as positive staining. Specimens were reviewed with staining intensity and staining extent. Staining intensity was rated as follows: negative (0), bordering (1), weak (2), strong (3). Staining extent was rated according to the percentage of positive cells in the field. Samples with no staining cells were rated as 0 and those with <25% of cells stained were rated as 1, those with 25 to 50% of cells stained were rated as 2, >50% of cells stained were rated as 3. The results of staining intensity and staining extent gave an overall staining score. The samples of staining where the score was 0 were marked as (–), those scored with 1 to 2 marked as (±), 3 to 4 marked as (+), and 5 to 6 marked as (++).

Statistical Analysis. The relationship between Aurora-A expression and pathologic characteristics was analyzed using a

χ^2 test with a statistical package for social scientists software (SPSS, Inc., Chicago, IL). Statistical significance was considered at the value of $P < 0.05$.

Immunofluorescence Staining. Slides were dewaxed in xylene, rehydrated in graded ethanol, and treated with heat in sodium citrate to recover antigenic property, which may be masked by formalin fixation and paraffin embedding. After blocking in 10% normal blocking serum at room temperature for 1 hour, slides were incubated with antibody to Aurora-A at 4°C overnight and then washed with PBS three times. Slides were then incubated with tetramethyl rhodamine isothiocyanate-conjugated antirabbit immunoglobulin G and followed by staining with 4',6-diamidino-2-phenylindole.

Protein Preparation from Tissue Samples and Western Blotting Assay. Frozen tissue samples were sectioned into small pieces and dissolved in lysis buffer containing 50 mmol/L Tris-Cl (pH 8.0), 150 mmol/L NaCl, 0.1% SDS, 100 $\mu\text{g/mL}$ phenylmethylsulfonyl fluoride, 2 $\mu\text{g/mL}$ aprotinin, 2 $\mu\text{g/mL}$ leupeptin, 1% NP40. The samples were homogenized, sonicated and kept on ice for 30 minutes. After centrifugation, the supernatant was collected for immunoblotting analysis. Briefly, 50 μg of cellular proteins were loaded onto a 10% SDS-PAGE. After electrophoresis, the proteins were transferred to Protran membranes. Membranes were blocked in 5% milk, washed with PBST (PBS with 0.1% Tween), and incubated with the indicated antibodies. After washing and incubation with horseradish peroxidase-conjugated antirabbit or antimouse antibody at 1:4,000 in 5% milk, the membranes were washed, and bound horseradish peroxidase was detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL) and exposed to X-ray film (35, 36).

Cell Culture. Human ESCC lines (KYSE-2, 9706, KYSE-450, KYSE-180, Colo-680, KYSE-150, KYSE-140, KYSE-410, KYSE-30, KYSE-510, T12, KYSE-70) were grown in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C under 5% CO_2 and saturated moisture.

Small Interfering RNA Transfection. The Aurora-A small interfering RNA (siRNA) sequence that we used is listed as the following: $^{155}\text{AUUCUCCCCAGCGCGUUSC}^{173}$. 9706 cells (2×10^5) were seeded onto 10-mm plates 16 hours before transfection. In each plate, 40 pmol of siRNA and 5 μL of Lipofectamine (Invitrogen, Carlsbad, CA) were added to 300 μL of Opti-MEM (Invitrogen) in separate tubes, mixed gently, and then incubated for 5 minutes at room temperature. After incubation, the siRNA and Lipofectamine solutions were mixed gently, allowed to sit 30 minutes at room temperature, diluted with 2.4 mL Opti-MEM, and added to the plates for 6 hours at 37°C. Equal volumes of medium with 10% FBS were added, and the plates were incubated for 24 to 72 hours until they were ready to assay for gene knockdown analysis.

Trans-Well Cell Migration Assay. Cell migration was examined with a chemotaxis chamber (Neuro Probe, Inc. Gaithersburg, MD). Cells (1×10^4) in 50 μL of culture medium were added to the upper chamber of the device, and the lower chamber was filled with 30 μL of medium containing 5 $\mu\text{g/mL}$ fibronectin (Sigma, St. Louis, MO). A polycarbonate membrane with a pore size of 8 μm was placed between the two chambers. The cells were allowed to migrate at 37°C in a 5% CO_2 humidified incubator for 6 hours. Nonmigrating cells on the upper

surface were carefully removed with a cotton swab. The filters were then fixed in methanol for 10 minutes and stained with Giemsa solution for 1 hour. Migrated cells on the membrane were counted under a microscope. Migration was quantified by counting the migrated cells in 10 random high-powered fields per filter.

RESULTS

Over-expression of Aurora-A in ESCC. To investigate whether Aurora-A abnormalities are linked to human ESCC, we first analyzed Aurora-A protein expression in tumor tissues. Totally, an immunohistochemical approach was used to collect and examine 64 clinical tumor specimens and 61 normal adjacent tissue samples. As shown in Fig. 1A, Aurora-A was found to be overexpressed in tumors (*c*, submucosal carcinoma; *d*, invasive carcinoma) compared with normal adjacent tissues (*a* and *b*). Similar observations were obtained by immunofluorescent staining (Fig. 1B). The results of immunohistochemistry were summarized in Table 1. Among 64 human ESCC samples, strong cytoplasmic staining (score between 3 and 6) of Aurora-A protein was detected in 45 tumors (69%). Weak or negative staining (score between 0 and 2) was detected in 20 tumors (31%). In contrast, of 61 normal adjacent tissues, only 1 sample exhibited strong staining for Aurora-A protein. The other 60 normal adjacent tissues presented weak or negative staining of Aurora-A protein.

In addition to immunohistochemical analysis, 20 tumor samples and their paired normal adjacent tissues were collected and assayed for Aurora-A expression by Western immunoblotting analysis. In agreement with the observations in the immunohistochemical assays, Aurora-A overexpression was detected by Western blot analysis in 12 of 20 tumors. In Fig. 2, representative results show increased levels of Aurora-A in human esophageal squamous cell carcinoma. Interestingly, some tumors displayed a >10 fold increase compared with their normal adjacent tissues.

Increased Expression of Aurora-A Correlates with Malignancy of ESCC. The association between Aurora-A expression and the degree of tumor differentiation and pathologic characteristics of tumors were examined. As summarized in

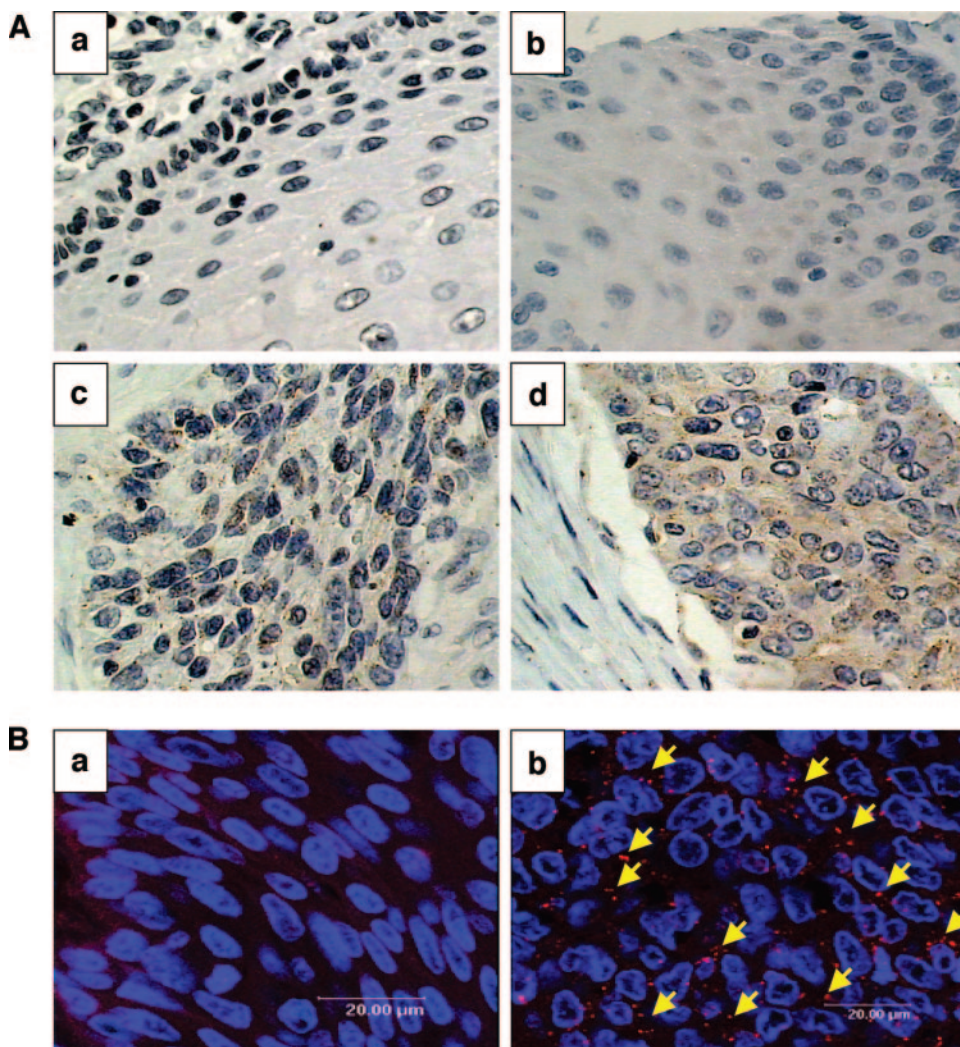


Fig. 1 Aurora-A expression in human ESCC and normal adjacent tissues. A, ESCC samples were collected and subjected to immunohistochemical staining with antibody to Aurora-A (see Materials and Methods). These tumor samples exhibited strong cytoplasmic staining of Aurora-A (*c* and *d*), but normal adjacent tissues showed no positive staining (*a* and *b*). Original magnification, $\times 400$. B, normal adjacent esophageal tissues (*a*) and esophageal tumor samples (*b*) were assayed by immunofluorescent staining. Aurora-A was visualized with antibody to Aurora-A and rhodamine-conjugated secondary antibody (red). Nuclei were labeled with DAPI (blue). Aurora-A protein was shown as red dot-like staining in cytoplasm (as pointed out by yellow arrows). The pictures shown in B (both *a* and *b*) were merges of Aurora-A (red) and nuclei staining (blue).

Table 1 Correlation of Aurora-A overexpression to malignant features of the tumor

	Tumors with Aurora-A overexpression			Total case	P value
	(-) or (±)	(+)	(++)		
In general					
Normal adjacent tissue	60	1		61	
Tumor tissue	20	25	20	64	<0.01 *
Grade of squamous cell carcinoma differentiation					
Good differentiation	5	5	4	14	
Moderate differentiation	13	18	10	41	<0.05 *
Poor differentiation	0	1	8	9	<0.01 ‡
Status of tumor malignancy by morphological examination					
Normal adjacent tissue	60	1		61	
Carcinoma <i>in situ</i>	19	21	1	41	<0.01 §
Invasive carcinoma	3	9	11	23	<0.01 ¶

* Normal adjacent tissue *versus* tumor tissue.

† Good differentiated tumor *versus* moderately differentiated tumor.

‡ Good differentiated tumor *versus* poorly differentiated tumor.

§ Normal adjacent tissue *versus* carcinoma *in situ*.

¶ Carcinoma *in situ* *versus* invasive carcinoma.

Table 1, tumors with moderate or poor differentiation exhibited higher expression levels of Aurora-A protein compared with tumors with good differentiation ($P < 0.05$ or $P < 0.01$). In addition, higher expression of Aurora-A was evidently seen in invasive ESCC ($P < 0.01$). Taken together, the extent of Aurora-A expression seems to be associated with malignancy of ESCC.

Association of Aurora-A Expression and Cell Migration in Human ESCC Cell Lines. We further examined Aurora-A expression in 12 human esophageal squamous cell carcinoma lines. As shown in Fig. 3A, these ESCC cell lines revealed variable levels of Aurora-A protein. There were >10-fold differences among these cell lines. However, the expression levels of Aurora-A did not correlate with cellular p53 mutations or Bcl-2 expression levels (results not shown). Next, we did cell *trans*-well assays to evaluate migration of these cell lines, because cell-migrating ability is closely associated with the potential of invasive growth. The measurement of each cell line for its migration was illustrated in Fig. 3B. Interestingly, it seemed that capabilities of those cells in migration were correlated with their Aurora-A levels. For example, 9706, which expresses the highest level of Aurora-A, exhibited the strongest migration. The cell lines (KYSE-450, KYSE-180, and Colo-680) expressing medium levels of Aurora-A showed moderate migration. In contrast, the cells (KYSE-140, KYSE-30, and KYSE-70) expressing low amounts of Aurora-A displayed weak migration. These results suggest that increased expression of Aurora-A might enhance migration of ESCC cells and contribute to the development of tumor invasive growth.

To further determine whether Aurora-A expression plays a role in ESCC cell migration, a RNA interference technique was used to knockdown endogenous Aurora-A. After introduction of Aurora-A siRNA into 9706 cells, which express the highest level of Aurora-A among all 12 ESCC lines, Aurora-A protein was shown to be substantially reduced at 48 and 72 hours after transfection. More than 60% of endogenous Aurora-A was suppressed by addition of Aurora-A siRNA. In contrast, a non-specific siRNA was included in the experiments and was shown to have no substantial effect on endogenous Aurora-A expres-

sion (Fig. 4A). We then used Aurora-A siRNA-treated 9706 cells for migration assay. In Fig. 4B, 9706 cells treated with Aurora-A siRNA for 48 or 72 hours exhibited greatly reduced migration compared with cells treated with nonspecific siRNA. In addition, we performed these same siRNA experiments in another esophageal cancer line KYSE-680, which also expresses a relatively higher level of Aurora-A, and obtained similar observations. Therefore, disruption of endogenous Aurora-A resulted in suppression of cell migration in ESCC cells. Collectively, Aurora-A seems to contribute to ESCC cell migration and may be involved in development of tumor invasion.

DISCUSSION

In this report, we have shown that Aurora-A, a centrosome-associated oncogenic protein, is overexpressed in human ESCC.

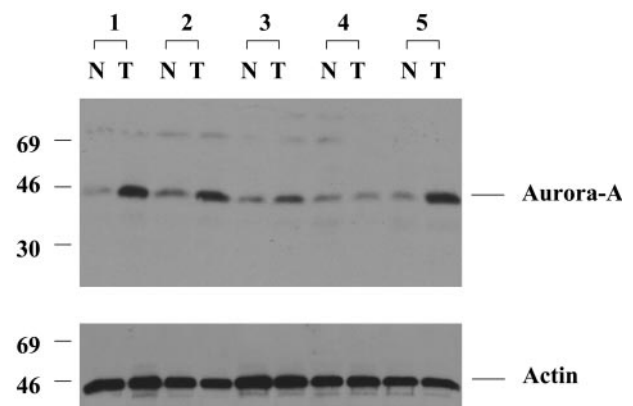


Fig. 2 Analysis of Aurora-A protein expression in human ESCC tissues by Western blot assay. Cell lysates were prepared from clinical tumors or normal adjacent tissues as described in Materials and Methods. One hundred micrograms of cellular protein was loaded onto 12% SDS polyacrylamide gels. After electrophoresis, the proteins were transferred to Immobilon membranes. Membranes were then blocked for 1 hour in 5% milk at room temperature and analyzed with antibodies to Aurora-A (top panel) and actin (bottom panel).

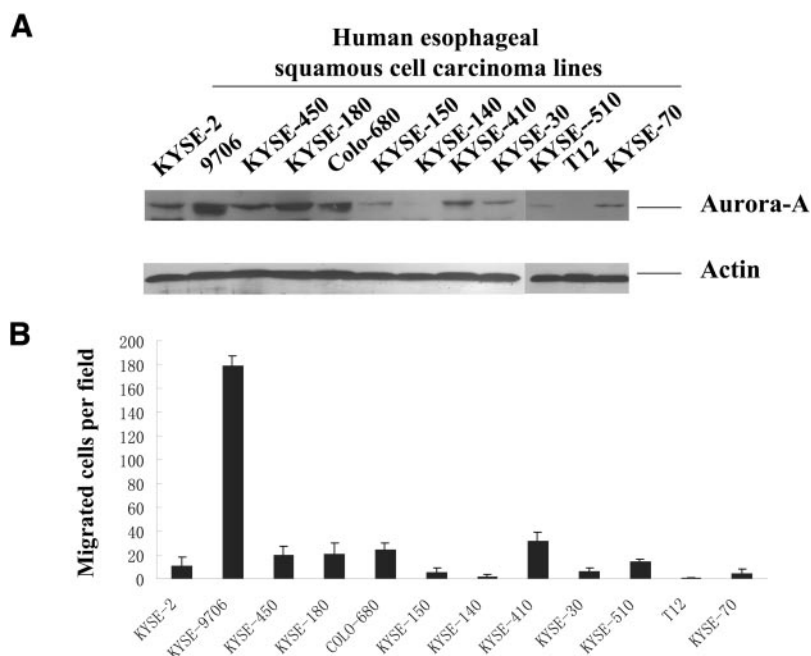


Fig. 3 A, expression of Aurora-A in ESCC cells. Human ESCC lines (KYSE-2, 9706, KYSE-450, KYSE-180, Colo-680, KYSE-150, KYSE-140, KYSE-410, KYSE-30, KYSE-510, T12, KYSE-70) were grown in RPMI 1640 supplemented with 10% fetal bovine serum. Cells were collected and washed three times with ice-cold PBS. Following preparation of cell lysates, 50 μ g of whole cell protein was used for immunoblotting analysis with anti-Aurora-A antibody as described in Fig. 2. B, analysis of cell migrating ability in human ESCC cells. Cells (1×10^4) from ESCC cell lines were used for *trans*-well migration assay (see Materials and Methods). Migration was quantified by counting the migrated cells in 10 random high-powered fields per filter.

Using an immunochemical staining approach, we found about 69% (45 of 64 samples) of ESCC tumor tissues to strongly overexpress Aurora-A protein. In contrast, <2% (1 of 61 samples) of normal adjacent tissues displayed high expression of Aurora-A (Fig. 1; Table 1). These findings were further confirmed by Western blot analysis (Fig. 2). Interestingly, overexpression of Aurora-A was shown to associate with the grades of tumor differentiation and invasive capability. Additionally, we have also examined Aurora-A expression and cell migration in 12 human ESCC lines and found that levels of Aurora-A protein were correlated to the migrating potentials of tumor cells. Moreover, disruption of endogenous Aurora-A protein through siRNA knockdown technique was shown to substantially suppress cell migration (Fig. 4), suggesting an association of Aurora-A with tumor cell invasive growth. Taken together, our studies show that Aurora-A, as an oncogenic protein, may play an important role in tumorigenesis and progression of ESCC.

Human ESCC is one of the leading causes of cancer death in the People's Republic of China (2). Because ESCC diagnosis is usually made in the advanced stages of the disease, the prognosis of ESCC is very poor. Currently, much effort is focused on developing novel approaches to early diagnosis and treatment of ESCC. To fulfill this purpose, a better understanding of the molecular mechanism of ESCC is urgently required. In the present study, we did a series of experiments to provide strong evidence that overexpression of Aurora-A could be one of the critical events in the multistage development of esophageal cancers. In addition, the findings presented in this report suggest that overexpression of Aurora-A possibly contributes to tumor invasive potentials, because disruption of endogenous Aurora-A in the ESCC lines (9706 and KYSE-680) by siRNA technique greatly reduced cell migrating ability (Figs. 3 and 4). This result indicates that overexpression of Aurora-A might

contribute to the malignancies (invasion and metastasis) of esophageal cancer. As an important kinase and cell cycle regulator, Aurora-A has been shown to interact with many important cellular proteins, including p53 and BRCA1 (21, 22), and disrupt/alter their physiological functions. The interactions of Aurora-A with those proteins may be associated with the role of Aurora-A in tumor progression, in addition to its role in tumor initiation (tumorigenesis). Interestingly, Miao *et al.* (34) have reported most recently that Phe311le polymorphism of Aurora-A is associated with the advanced disease status of ESCC. This result supports our conclusion that abnormalities contribute to the development of ESCC. Therefore, Aurora-A may possibly serve as a useful molecule for evaluating advanced stages of ESCC and determining the prognosis of this disease. Furthermore, Aurora-A might also be an attractive candidate for developing a gene-knockdown approach to ESCC therapy (37).

Future investigations are needed to explore the precise mechanism by which Aurora-A plays a role in ESCC occurrence and progression. Most likely, Aurora-A-induced chromosomal aberrations and genomic instability may greatly contribute to its oncogenic properties. Aurora-A inhibition of p53 and BRCA1 may also account for its cell transforming capabilities. As discussed earlier, mutations of the tumor suppressor p53, loss of expression of the Rb gene, and amplifications of Myc, EGFR, and cyclin D1 are often found to associate with ESCC development (8–11). Therefore, it is possible that Aurora-A protein acts synergistically with altered gene expression in ESCC development.

In summary, we have reported that expression of Aurora-A protein is highly increased in ESCC. Overexpression of Aurora-A is associated with the grades of tumor differentiation and the malignant status of tumors. The findings in this study pro-

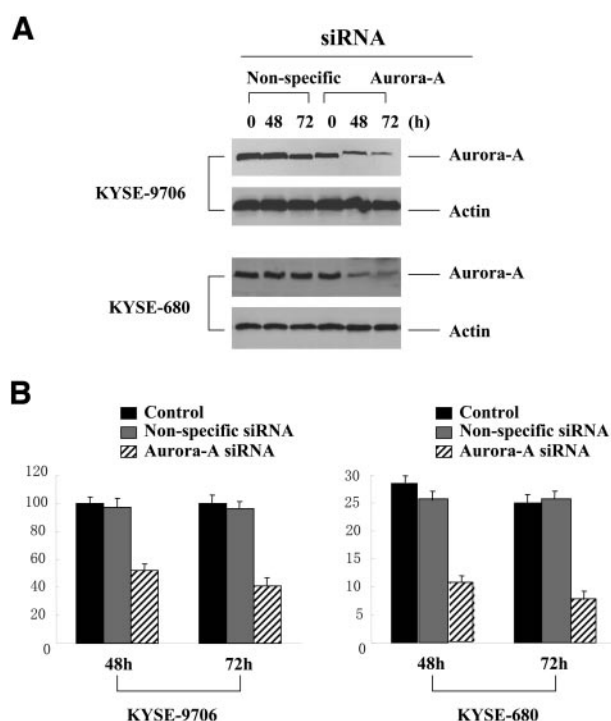


Fig. 4 Effect of Aurora-A siRNA on endogenous Aurora-A expression in ESCC cells. **A**, 2×10^5 of 9706 and KYSE-680 cells were plated onto 100-mm dishes and subjected to Aurora-A siRNA transfection. After incubation with siRNA, cells were collected at the indicated time and followed by Western blot analysis. **B**, effect of disrupted Aurora-A expression on cell-migrating ability. Following transfection with Aurora-A siRNA, 9706 or KYSE-680 cells were collected at 48- and 72-hour time points for *trans*-well assay. Migrations were measured as described in Fig. 3.

vide new insights into understanding the molecular mechanism involved in ESCC occurrence and progression and may lead to the development of new approaches for effective diagnosis and therapy.

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