The Clinical Significance of Aurora-A/STK15/BTAK Expression in Human Esophageal Squamous Cell Carcinoma

Eiji Tanaka,[1] Yosuke Hashimoto,[1] Tetsuo Ito,[1] Tomoyuki Okumura,[1] Takatsugu Kan,[1] Go Watanabe,[1] Masayuki Imamura,[2] Johji Inazawa,[3] and Yutaka Shimada[1]

[1]Department of Surgery and Surgical Basic Science, Graduate School of Medicine, Kyoto University, Kyoto, Japan, [2]Department of Surgery Saiseikai Noe Hospital, Osaka, Japan; and [3]Department of Molecular Cytogenetics, Graduate School of Biomedical Science, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan

ABSTRACT

Purpose: ***Aurora-A/STK15/BTAK (Aurora-A)*** **encodes a Serine/Threonine kinase associated with chromosomal distribution, and its up-regulation induces chromosomal instability thereby leading to aneuploidy and cell transformation in several types of cancer. In this study, we investigated the role of Aurora-A in human esophageal squamous cell carcinoma (ESCC).**

Experimental Design: **The expression levels of Aurora-A mRNA were compared in 33 ESCC tissues with that in corresponding normal esophageal epithelium by semiquantitative reverse transcription-PCR, and the distribution patterns and expression levels of Aurora-A protein were immunohistochemically investigated in the ESCC tumors of 142 patients. The results were then separately compared with the clinicopathologic findings of the patients, and the expression of Aurora-A was examined in nine ESCC cell lines and a normal esophageal epithelial cell line using Western blot analysis.**

Results: **The up-regulation of Aurora-A mRNA was found in 30% (10 of 33) of the tumors by semiquantitative reverse transcription-PCR, and protein up-regulation was found in 53% (75 of 142) of the patients by immunohistochemistry. mRNA and protein up-regulation of Aurora-A were correlated with distant lymph node metastasis (P = 0.05 and P = 0.04, respectively), and patients with Aurora-A mRNA or protein up-regulation had a poorer prognosis (P = 0.003 and P = 0.0009, respectively). Furthermore, multivariate analysis revealed that up-regulation of the Aurora-A protein was an independent prognostic factor. In addition, Aurora-A expression in all ESCC cell lines was higher than that in a normal esophageal epithelial cell line.**

Conclusions: **The up-regulation of Aurora-A expression may reflect the malignant behavior of ESCC and may prove useful information as a prognostic factor for ESCC patients.**

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Requests for reprints: Yutaka Shimada, Department of Surgery and Surgical Basic Science, Graduate School of Medicine, Kyoto University, 54 Shogoin Kawara-Cho, Sakyo-Ku, Kyoto, 606-8507, Japan. Phone: 81-75-751-3626; Fax: 81-75-751-4390; E-mail: shimada@kuhp.kyoto-u.ac.jp.

INTRODUCTION

During the proliferation of normal cells, the centrosome ensures the equal segregation of chromosomes to the postmitotic daughter cells by organizing the bipolar mitotic spindle. In contrast, in cancer cells multipolar mitotic spindles and various centrosomal anomalies, such as supernumerary centrosomes, centrosomes of abnormal size and shape, aberrantly phosphorylated centrosome proteins, and premaurely split centrosomes are frequently observed (1–7). It is conceivable that such abnormalities disrupt normal chromosomal segregation, producing aneuploid cells. A recently cloned and characterized mitotic kinase-encoding gene, *Aurora-A/STK15/BTAK* (8, 9), has been implicated in the regulation of centrosome duplication and is frequently amplified/overexpressed in human tumors (9, 10).

Aurora-A is a member of the Aurora/Ipl1p family of cell cycle–regulating Serine/Threonine kinases and is localized at interphase and mitotic centrosomes and the spindle poles in the nucleus where it regulates proper chromosome segregation and cytokinesis. Recent studies have shown that the ectopic expression of Aurora-A in mouse NIH/3T3 cells and Rat 1 fibroblasts causes centrosome amplification and transformation *in vitro* as well as tumorgenesis *in vivo* (9, 10). Furthermore, the up-regulation of Aurora-A in diploid human breast epithelial cells leads to abnormal centrosome numbers and the induction of aneuploidy (9). A correlation between the up-regulation of Aurora-A and clinical aggressiveness has also been described for several cancers (11–14). These findings suggest that *Aurora-A* is a critical kinase-encoding gene whose up-regulation leads to centrosome amplification, chromosomal instability, and potentially, oncogenesis.

The *Aurora-A* gene is localized on chromosome 20q13, an area amplified in a variety of human cancers (15–17). Using comparative genomic hybridization, we previously investigated copy number aberrations in 29 esophageal squamous cell carcinoma (ESCC) cell lines, and found that a chromosome gain of the proximal part of 20q is one of the most common copy numbers (19 of 29, 65.5%; ref. 18). In this study, to clarify the clinical significance of Aurora-A expression in ESCC patients and the role of Aurora-A in human ESCCs, we examined the expression of Aurora-A mRNA and protein in ESCC tissues as well as cell lines and analyzed the clinicopathologic features of Aurora-A expression in ESCC patients.

MATERIALS AND METHODS

Tumor Samples and Cell Culturing. Frozen tumor tissues and their corresponding normal tissues were obtained for semiquantitative reverse transcription-PCR (RT-PCR) from 33

patients, and paraffin-embedded sections were acquired from 142 patients who underwent surgery at Kyoto University Hospital from 1990 to 2001 for immunohistochemistry using primary ESCC. All tumors were confirmed as being ESCC by the Clinicopathologic Department of the hospital. The cases were diagnosed as formalin-fixed, paraffin-embedded, H&E-stained representative specimens and were classified according to the 5th edition of the tumor-node-metastasis classification (19, 20). Thus, lymph node metastasis observed outside the regional lymph node was classified as "distant lymph node metastasis." All M1a and M1b cases exhibited regional lymph node metastasis (N1), and there was no distant organ metastasis observed for any case. These tumors were excluded from this study, and there were no operative mortalities. In addition, all preoperative chemotherapy was cisplatin-based. The tumor characteristics for semiquantitative RT-PCR are summarized in Table 1. Their median follow-up for survival was 31.3 months. The tumor characteristics for immunohistochemistry are summarized in Table 2, for which the median follow-up was 32.6 months. Information regarding patient gender, age, stage of the disease, preoperative chemotherapy, and histopathologic factors was extracted from past medical records.

Written informed consent was obtained from the patients regarding the performance of surgery and the use of resected samples for research. The approval numbers of the Institutional Review Board of Kyoto University are 232 and G48.

All tested ESCC cell lines of the KYSE series were established in our laboratory and maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD) and Ham's F12 (Nissui Pharmaceutical, Tokyo, Japan) mixed (1:1) medium containing 2% fetal bovine serum (21). The normal esophageal epithelial cell line NEK2 was established in our laboratory and maintained in keratinocyte serum-free medium containing 2.5 μg of epidermal growth factor and 25 μg of bovine pituitary extract (22). HeLa cells were purchased from the American Type Culture Collection (Rockville, MD), cultured in DMEM (Life Technologies) with 10% FCS and used as a positive control (23, 24).

Table 1 Patient and tumor characteristics for semiquantitative RT-PCR

Total	33 (%)
Mean age (range), y	63.0 (47-84)
<63	18 (54.5)
≥63	15 (45.5)
Gender	
Male	29 (87.9)
Female	4 (12.1)
Extent of the primary tumor	
T1, T2	7, 6 (21.2, 18.2)
T3, T4	12, 8 (36.4, 24.2)
Lymph node metastasis	
N0	10 (30.3)
N1	23 (69.7)
Distant lymph node metastasis	
M0	27 (81.8)
M1a, M1b	4, 2 (12.1, 6.1)
Stage	
I, IIa, IIb	5, 4, 3 (15.2, 12.1, 9.0)
III, IVa, IVb	15, 1, 5 (45.5, 3.0, 15.2)
Preoperative chemotherapy	
Performed	17 (51.5)
Not performed	16 (48.5)

Table 2 Patient and tumor characteristics for immunohistochemistry

Total	142 (%)
Mean age (range), y	64.0 (48-90)
<64	61 (43.0)
≥64	81 (57.0)
Gender	
Male	118 (83.1)
Female	24 (16.9)
Extent of the primary tumor	
T1, T2	47, 23 (33.1, 16.2)
T3, T4	44, 28 (31.0, 19.7)
Lymph node metastasis	
N0	56 (39.4)
N1	86 (60.6)
Distant lymph node metastasis	
M0	119 (83.8)
M1a, M1b	13, 10 (9.2, 7.0)
Stage	
I, IIa, IIb	32, 20, 24
III, IVa, IVb	42, 7, 17 (29.6, 4.9, 12.0)
Preoperative chemotherapy	
Performed	49 (34.5)
Not performed	93 (65.5)

Purification of Total Cellular RNA and Semiquantitative Reverse Transcription-PCR. Total cellular RNA was purified from frozen stored tissues of ESCC patients by the TRIzol reagent (Invitrogen, Carlsbad, CA) method (25, 26). Reverse transcription of total cellular RNA (5 μg) was done for each sample using a First-Strand cDNA Synthesis Kit (Amersham, Buckinghamshire, United Kingdom), and cDNA was subjected to PCR for 25 cycles of amplification using an Advantage cDNA PCR kit (Becton Dickinson Biosciences, Palo Alto, CA). Amplification was done for 30 seconds at 94°C and 30 seconds at 54°C, and the final extension step was carried out for 5 minutes at 72°C. The amplification products were separated on 1.5% agarose gels and visualized by ethidium bromide staining. The PCR primers used for Aurora-A were 5′-GGCAAGAGAAAAGCAAAGC-3′ for the forward primer and 5′-ATCATTTCAGGGGGCAGGTA-3′ for the reverse primer. For glyceraldehyde-3-phosphate dehydrogenase, the forward primer used was 5′-TGGTATCGTGGAAGGACTCATGAC-3′ and the reverse primer used was 5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′. For the positive controls, cDNA from HeLa cells was used for each analysis.

Immunohistochemical Staining. Resected esophageal specimens were fixed in 10% formaldehyde and then embedded in paraffin within 4 days. Sections were cut and mounted on aminopropyltriethoxysilane-coated glass slides, and immunohistochemical staining was done using an Envision + kit (Dako Cytomation, Glostrup, Denmark). Briefly, the tissue sections were deparaffinized and rehydrated in water, after which antigen retrieval was carried out by incubation in Target retrieval solution (Dako Cytomation) buffer at 121°C for 5 minutes in an autoclave. Endogenous peroxidase and nonspecific antibody reactivity was blocked with peroxidase blocking reagent (Dako Cytomation) at room temperature for 15 minutes. The sections were then incubated overnight at 4°C with anti-human Aurora-A polyclonal antibody (Trans Genic Inc., Kumamoto, Japan. diluted 1:100) in PBS containing 1% bovine serum albumin. After next rinsing in TBS containing

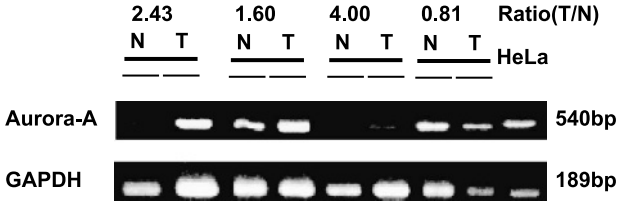


Fig. 1 Semiquantitative RT-PCR of ESCC tissues. Aurora-A mRNA expression in tumor samples (*T*) and their corresponding normal epithelium (*N*) were investigated. mRNA from HeLa cells was used in the same analysis as a positive control. The signal intensity of each sample was calculated using NIH image software, and the ratio Aurora-A/GAPDH was then scored. Next, Aurora-A expression in each specimen was evaluated using the ratio of tumor/corresponding normal epithelial part (T/N ratio), and using this result the patients were divided into two groups.

1% Tween 20, the sections were incubated with anti-rabbit labeled polymer horseradish peroxidase for 30 minutes at room temperature, and after rinsing again, they were incubated with 3,3′-diaminobenzidine liquid system (Dako Cytomation) for 5 minutes, counterstained with Mayer's hematoxylin, dehydrated, and then mounted. The immunoreactivity of each slide was confirmed using anti-human cytokeratin antibody (Dako Cytomation, clone MNF 116; see supplemental data).

Evaluation of Immunohistochemical Staining. A section without primary antibody was used as a negative control for each case. Positive staining of the nucleus was evaluated in five areas of each section. Each sample was divided into two groups according to the percentage of Aurora-A nuclear staining–positive cells among the tumor cells, for which we classified tumors as positive when >30% nucleus of the tumor was stained.

All slides were independently evaluated by two investigators (E.T. and Y.H.) without prior knowledge of each patient's clinical information. When the opinions of the two evaluators were different, agreement was reached by careful discussion. The reproducibility of the evaluations of each investigator was tested via second assessment for all cases.

Table 3 The relationship between Aurora-A mRNA expression and patient clinicopathologic characteristics

Status of Aurora-A expression	Negative (n = 23), ratio < 2.0	Positive (n = 10), ratio ≥ 2.0	*P
Age (y)			0.07
<63	10	8	
≥63	13	2	
Gender			0.07
Male	22	7	
Female	1	3	
Extent of the primary tumor			0.70
T1, T2	10 (4, 6)	3 (3, 0)	
T3, T4	13 (10, 3)	7 (2, 5)	
Lymph node metastasis			0.68
N0	8	2	
N1	15	8	
Distant lymph node metastasis			0.05
M0	21	6	
M1a, M1b	2 (0, 2)	4 (4, 0)	
Stage			0.26
I, IIa, IIb	10 (3, 4, 3)	2 (2, 0, 0)	
III, IVa, IVb	13 (11, 0, 2)	8 (4, 1, 3)	

*Fisher's exact test.

Statistical Analyses. Cumulative survival analysis and disease-free survival analysis were done using the Kaplan-Meier method and analyzed by the log-rank test. Multivariate analysis was done using the Cox's regression model and logistic multivariate regression model. The correlation between Aurora-A expression and each clinicopathologic factor was evaluated by Fisher's exact test. Each statistical analysis was done using StatView 4.5 (Abacus Concept, Inc., Berkley, CA). A $P < 0.05$ was considered to indicate statistical significance.

Western Blot Analysis. Cells were washed with PBS and treated with lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, and 1% Triton X-100] containing Complete Mini protease inhibitor (Roche Diagnostics, Mannheim, Germany) on ice for 15 minutes and then centrifuged. Next, the protein content was measured using a bicinchoninic acid protein assay reagent (Pierce, Rockford, MA) after which the cell lysates (50 μg) were electrophoresed

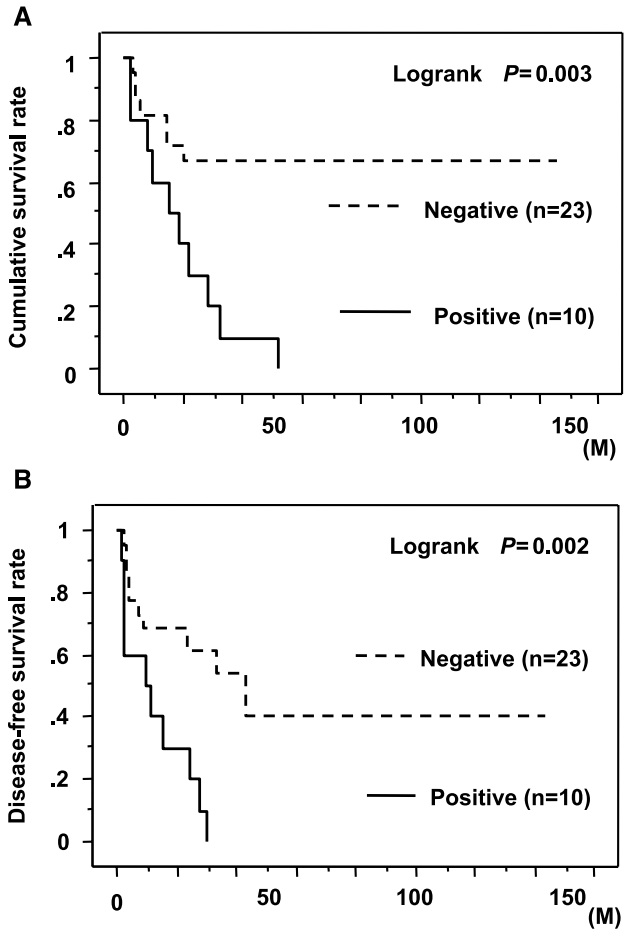
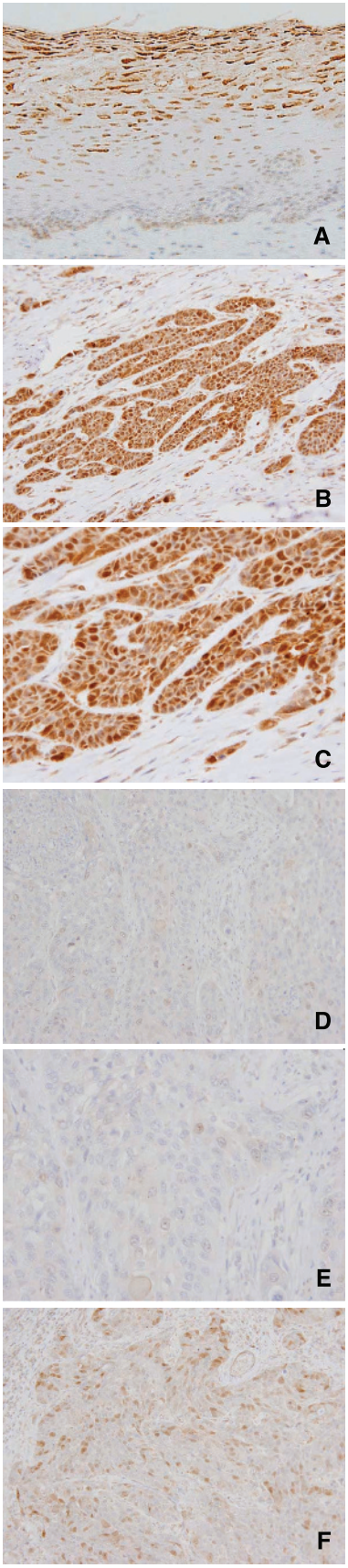


Fig. 2 The association between Aurora-A mRNA expression and patient prognosis. *A,* cumulative survival rate in relation to Aurora-A mRNA expression. The cumulative survival rate of patients with Aurora-A mRNA–positive tumors was lower than that of patients with Aurora-A-negative tumors. Positive: T/N ratio > 2.0. Negative: T/N ratio < 2.0. We classified 10 tumors as positive and 23 as negative. *B,* disease-free survival rate in relation to Aurora-A mRNA expression. The disease-free survival rate of patients with Aurora-A mRNA–positive tumors was lower than that of patients with Aurora-A-negative tumors. Positive: T/N ratio > 2.0. Negative: T/N ratio < 2.0.



on 2% to 15% gradient polyacrylamide gel (Daiichi Pure Chemicals, Tokyo, Japan) and transferred to a polyvinylidene difluoride membranes (Millipore, Bedford, MA) using a semidry transfer blot system (Bio-Rad, Hercules, CA). After blocking with TBS containing 1% Tween 20 and 5% skimmed milk for 1 hour, the membranes were incubated at 4°C over night with anti-human Aurora-A polyclonal antibody (Trans Genic, diluted 1:100) or anti-human β-actin monoclonal antibody (Sigma Inc., St. Louis, MO; diluted 1:2000), washed, and incubated with horseradish peroxidase-labeled anti-rabbit or anti-mouse IgG (Zymed, San Francisco, CA) as a secondary antibody. Proteins were detected using Western Blotting Luminol Reagent (Santa Cruz Biotechnology, San Diego, CA).

RESULTS

Semiquantitative Reverse Transcription-PCR. The signal intensity of each sample was calculated using NIH image (NIH, Bethesda, MD), and the ratio of Aurora-A/glyceraldehyde-3-phosphate dehydrogenase was then scored. Next, Aurora-A expression in each specimen was then evaluated by the ratio of tumor / corresponding normal epithelial part (T/N ratio), and divided into four subgroups according to the T/N ratio.

Parts of the results are shown in Fig. 1. The distribution of Aurora-A mRNA expression among 33 patients was as follows: 14 tumors had a T/N ratio of 0 to 1.0, 4 a ratio of 1.0 to 1.5, 5 a ratio of 1.5 to 2.0, and 10 a ratio of >2.0. The median T/N ratio was 1.45, and we classified the tumors with a T/N ratio over 2.0 as positive, whereas the tumors with a ratio of <2.0 were classified as negative (Fig. 1).

Correlation between Aurora-A mRNA Expression and Clinicopathologic Findings. Among the 33 patients, positive Aurora-A expression was correlated with distant lymph node metastasis ($P = 0.05$). However, it was not correlated with age ($P = 0.07$), gender ($P = 0.07$), extent of the primary tumor ($P = 0.70$), regional lymph node metastasis ($P = 0.68$), and the tumor-node-metastasis, system for staging cancer stage ($P = 0.26$; Table 3).

Association between Aurora-A mRNA Expression and Patient Prognosis. Overall, the cumulative survival rate of the patients with Aurora-A-positive tumors was significantly lower than that of the patients with Aurora-A-negative tumors ($P = 0.003$; Fig. 2*A*). The disease-free survival rate of the patients with Aurora-A-positive tumors was also significantly lower ($P = 0.002$; Fig. 2*B*).

Fig. 3 Immunohistochemical staining of Aurora-A. *A,* staining pattern of normal parts. We observed diffuse cytoplasmic staining in the horny layer, nuclear staining in the granular layer, no staining in the spindle layer, and weak cytoplasmic and moderate nuclear staining in the basal layer. *B* and *C,* positive staining for Aurora-A expression. Positive tumors showed strong nuclear staining, and occasional cytoplasmic staining was also observed. *D* and *E,* negative staining pattern for Aurora-A expression. Negative tumors did not show nuclear staining. *F,* this tumor did not show cytoplasmic staining, but did exhibit nuclear staining. We classified it as positive according to its nuclear-staining rate, which was 38.8%. (Original magnification: *A*, *B*, *D*, and *F* at ×200; *C* and *E* at ×400).

Table 4 The relationship between Aurora-A protein expression and patient clinicopathologic characteristics

Status of Aurora-A expression	Negative (n = 67), nuclear staining <30%	Positive (n = 75), nuclear staining >30%	*P
Age (y)			0.31
<64	32	29	
64	35	46	
Gender			0.51
Male	54	64	
Female	13	11	
Extent of the primary tumor			0.24
T1, T2	37 (26, 11)	33 (21, 12)	
T3, T4	30 (21, 9)	42 (23, 19)	
Lymph node metastasis			0.13
N0	31	25	
N1	36	50	
Distant lymph node metastasis			0.04
M0	61	58	
M1a, M1b	6 (2, 4)	17 (10, 7)	
Stage			0.18
I, IIa, IIb	40 (17, 12, 11)	36 (15, 8, 13)	
III, IVa, IVb	27 (20, 1, 6)	39 (22, 6, 11)	

*Fisher's exact test

Immunohistochemical Staining Patterns. The findings for semiquantitative RTPCR led us to speculate that up-regulation of the Aurora-A protein in the surgical specimens might have occurred. For the immunohistochemical study, we first examined the staining pattern of Aurora-A protein in the specimens. Preliminary data for immunofluorescent staining of Aurora-A in HeLa cells showed heterogeneous nuclear staining

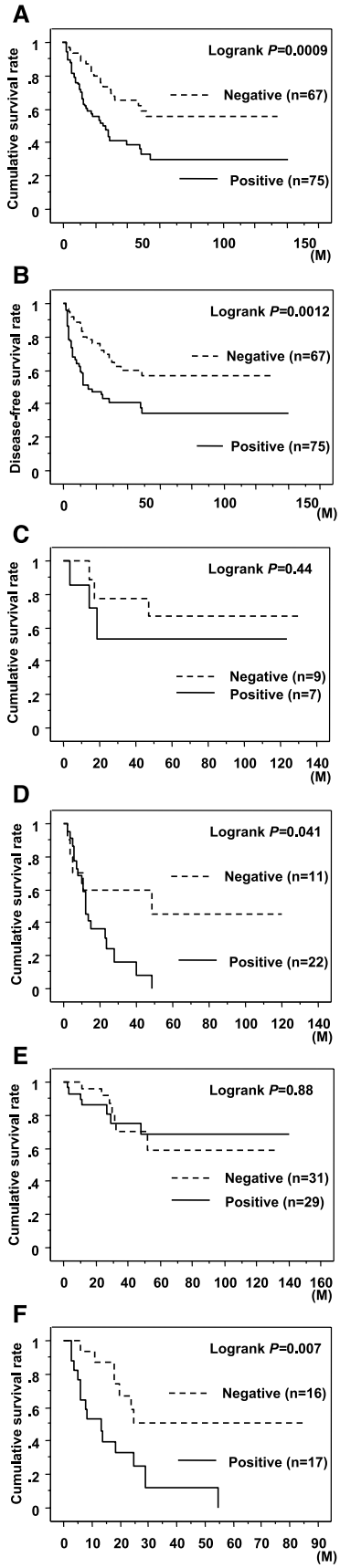


Fig. 4 The association between Aurora-A protein expression and patient prognosis. *A,* cumulative survival rate in relation to Aurora-A immunohistochemical expression. The cumulative survival rate of patients with immunohistochemically Aurora-A-positive tumors was lower than that of patients with Aurora-A-negative tumors. Positive: nuclear-staining ratio >30%. Negative: nuclear-staining ratio <30%. We classified 75 tumors as positive and 67 as negative. *B,* disease-free survival rate in relation to Aurora-A immunohistochemical expression. The disease-free survival rate of patients with immunohistochemically Aurora-A-positive tumors was lower than that of patients with Aurora-A-negative tumors. Positive: nuclear-staining ratio >30%. Negative: nuclear-staining ratio <30%. *C,* cumulative survival rate in relation to Aurora-A immunohistochemical expression among patients who received preoperative chemotherapy at stage I or II. The cumulative survival rate of patients with immunohistochemically Aurora-A-positive tumors did not show statistical significance. *D,* cumulative survival rate in relation to Aurora-A immunohistochemical expression among patients who received preoperative chemotherapy at stage III or IV. The cumulative survival rate of patients with immunohistochemically Aurora-A-positive tumors was lower than that of patients with Aurora-A-negative tumors. *E,* cumulative survival rate in relation to Aurora-A immunohistochemical expression among patients who did not receive preoperative chemotherapy at stage I or II. The cumulative survival rate of patients with immunohistochemically Aurora-A-positive tumors did not show statistical significance. *F,* cumulative survival rate in relation to Aurora-A immunohistochemical expression among the patients who did not receive preoperative chemotherapy at stage III or IV. The cumulative survival rate of patients with immunohistochemically Aurora-A-positive tumors was lower than that of patients with Aurora-A-negative tumors.

Table 5 Logistic regression model

Terms	Odds ratio	95% Confidence interval	*P*
Gender (male)		0.60-3.93	0.38
Age (>64 y)	0.75	0.37-1.48	0.40
Extent of the primary tumor (T3, T4)	1.28	0.62-2.60	0.50
Lymph node metastasis (N1)	1.24	0.59-2.65	0.57
Distant lymph node metastasis (M1a, M1b)	2.61	0.92-8.23	0.08

NOTE. Aurora-A expression.

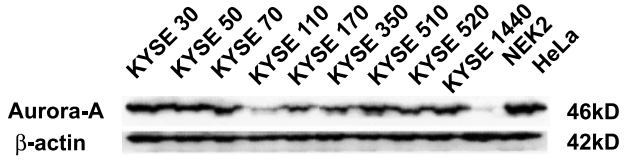


Fig. 5 Western blot analysis of ESCC cell lines (KYSE series) and the normal epithelium cell line NEK2. Aurora-A expression in the KYSE cell lines was higher than in the normal esophageal epithelial cell line. HeLa cells were included as a positive control in the same analysis.

and a high concentration in the centrosome (data not shown), and therefore we then examined the nuclear staining of the surgical specimens. For normal esophageal epithelium, we observed diffuse cytoplasmic staining in the horny layer, nuclear staining in the granular layer, no staining in the spindle layer, and weak cytoplasmic and moderate nuclear staining in the basal layer (Fig. 3*A*). In contrast, the tumors often showed strong nuclear staining compared with the normal epithelium. Occasional cytoplasmic staining was also observed in the tumors, but we classified them only according to the percentage of nuclear staining. A representative tumor with intense staining due to Aurora-A expression in the nucleus is shown in Fig. 3*B* and *C*. On the other hand, some specimens did not show nuclear staining (Fig. 3*D* and *E*). We scored the percentage of nuclear staining–positive cells among the tumor cells and the distribution of Aurora-A immunohistochemical expression among 142 patients as follows: 17 tumors were <10%; 26 tumors, 10% to 20%; 24 tumors, 20% to 30%; 27 tumors, 30% to 40%; 28 tumors, 40% to 50%; 8 tumors, 50% to 60%; and 12 tumors were >80%. For these specimens, the median nuclear staining percentage was 33.9. A sample case which scored 38.8% is shown in Fig. 3*F*. We classified 75 tumors with staining >30% of the nucleus as positive, whereas 67 tumors with staining <30% were classified as negative.

Correlation between Aurora-A Immunohistochemical Expression and Clinicopathologic Findings. Among the 142 patients, positive Aurora-A expression was correlated with distant lymph node metastasis ($P = 0.04$). However, it was not correlated with gender ($P = 0.51$), age ($P = 0.31$), extent of the primary tumor ($P = 0.24$), regional lymph node metastasis ($P = 0.13$), and the tumor-node-metastasis stage ($P = 0.18$; Table 4). In addition, we analyzed the Aurora-A-positive staining using a logistic multivariate regression model and found that distant lymph node metastasis (M) made the most contribution to Aurora-A protein expression (odds ratio, 2.61; 95%confidence interval, 0.92-8.23; $P = 0.08$) compared with the other factors (Table 5).

Table 6 Cox's multivariate analysis

Terms	Risk ratio	95% Confidence interval	*P*
Gender (male)	1.41	0.69-2.90	0.35
Age (>64 y)	1.33	0.79-2.23	0.29
Extent of the primary tumor (T3, T4)	1.71	1.00-2.94	0.05
Lymph node metastasis (N1)	3.01	1.53-5.94	0.001
Distant lymph node metastasis (M1a, M1b)	1.64	0.89-3.00	0.11
Preoperative chemotherapy (+)	1.24	0.72-2.11	0.44
Aurora-A (immunohistochemically positive)	1.98	1.15-3.41	0.01

Association between Aurora-A Immunohistochemical Expression and Patient Prognosis. Overall, the cumulative survival rate of the patients with Aurora-A-positive tumors was significantly lower than that of the patients with Aurora-A-negative tumors ($P = 0.0009$; Fig. 4*A*). The disease-free survival rate of the patients with Aurora-A-positive tumors was also significantly lower ($P = 0.0012$; Fig. 4*B*). Univariate analysis showed that the extent of the primary tumor (risk ratio, 2.33; $P = 0.0013$), regional lymph node metastasis (risk ratio, 4.44; $P < 0.0001$), distant lymph node metastasis (risk ratio, 2.84; $P = 0.0002$), and Aurora-A status (risk ratio, 2.33; $P = 0.0013$) were statistically significant prognostic factors. Among the patients, 23 with distant lymph node metastasis had the poorest prognosis ($P = 0.019$; see supplemental data). Moreover, we divided the patients into four subgroups according to whether or not they received preoperative chemotherapy and their tumor-node-metastasis stage, and analyzed each subgroup. Regardless of receiving preoperative chemotherapy or not, among the patients at stage III or IV, the cumulative survival rate of those with immunohistochemically Aurora-A-positive tumors was lower than that of those with Aurora-A-negative tumors (Fig. 4*D* and *F*; $P = 0.041$ and $P = 0.007$, respectively). On the other hand, regardless of receiving preoperative chemotherapy or not, among the patients at stage I or II, the cumulative survival rate for those with Aurora-A-positive tumors was not statistically significant (Fig. 4*C* and *E*; $P = 0.44$ and $P = 0.88$, respectively). Finally, Cox's multivariate analysis revealed that the extent of the primary tumor ($P = 0.05$), lymph node metastasis ($P = 0.001$), and Aurora-A status ($P = 0.01$) were independent prognostic factors (Table 6).

Western Blot Analysis. We tested for Aurora-A protein expression in the cell lines. Western blot analysis showed Aurora-A expression in the ESCC cell lines (KYSE series), and Aurora-A expression in the KYSE cell lines was higher than that in the normal esophageal epithelial cell line NEK2 (Fig. 5).

DISCUSSION

The results for semiquantitative RT-PCR and immunohistochemistry showed that mRNA and protein up-regulation of Aurora-A frequently occur in ESCC and contribute to a poor prognosis. In addition, Aurora-A protein was overexpressed in all ESCC cell lines compared with the normal esophageal epithelial cell line. These findings suggest that

Aurora-A up-regulation is a common abnormality in ESCC and may play a role in its progression.

Aurora-A status and distant lymph node metastasis was found to be significantly associated using Fisher's exact test, and similarly, logistic regression analysis showed a tendency toward their association, albeit not statistically significant. Several previous reports showed that the up-regulation of Aurora-A is correlated with these malignant phenotypes in many cancers (11–14). The up-regulation of Aurora-A leads to centrosome amplification and consequently causes chromosome instability (9), which may help tumor cells obtain invasive and metastatic phenotypes. In addition, a more recent report showed that the Aurora-A Phe31Ile polymorphism is associated with advanced stage ESCC and its occurrence (27). For esophageal carcinoma, when a patient has distant lymph node metastasis, it can be considered a systemic disease (20). We think that some biological changes might occur at this time. The number of patients who had distant lymph node metastasis in our study was small, but we speculate that the excessive expression of Aurora-A may be related to the progression from a regional disease state to a systemic disease state for ESCC. Furthermore, not only the up-regulation of Aurora-A but also the frequent change in Aurora-A polymorphism may affect the invasiveness and metastatic properties of tumor cells in ESCC.

With regard to the correlation between immunohistochemical Aurora-A staining and the prognosis of ESCC, the cumulative survival rate and disease-free survival rate of patients with Aurora-A-positive tumors was significantly lower than that of patients with Aurora-A-negative tumors. Thus, an Aurora-A-positive status is an independent prognostic factor, implying that the elevated expression of Aurora-A may be an indicator of the patient's prognosis. Recent reports showed that the up-regulation of Aurora-A results in resistance to apoptosis induced by paclitaxel in a human cancer cell line (28, 29). This raises the possibility that Aurora kinase inhibition may provide for a new approach for the treatment of multiple human malignancies (30). Currently, additional studies including those examining the association between Aurora-A expression status and chemosensitivity of ESCC are under way in our laboratory.

In conclusion, our findings suggest that mRNA and the immunohistochemical up-regulation of Aurora-A may be useful as a prognostic factor for ESCC patients. Future studies on the physiologic targets of Aurora-A and its potential role in the pathogenesis of ESCC will be helpful for finding a novel therapeutic strategy for the treatment of ESCC.

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