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# Two Functional Coding Single Nucleotide Polymorphisms in STK15 (Aurora-A) Coordinately Increase Esophageal Cancer Risk

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## Abstract

STK15/Aurora-A is a serine/threonine kinase essential for chromosome segregation and cytokinesis, and is considered to be a cancer susceptibility gene in mice and humans. Two coding single nucleotide polymorphisms in Aurora-A, 91T>A [phenylalanine/isoleucine (F/I)] and 169G>A [valine/isoleucine (V/I)], create four haplotypes, 91T-169G, 91A-169G, 91T-169A, and 91A-169A. We evaluated the association between these coding single nucleotide polymorphisms and esophageal cancer risk by genotyping 197 esophageal cancer cases and 146 controls. Haplotype 91A-169A (I31/I57) was observed to be statistically more frequent in cancer cases (odds ratio, 3.1452; 95% confidence interval, 1.0258–9.6435). Functional differences among the four isoforms were then analyzed to reveal the source of the cancer risk. Kinase activity levels of I31/I57 and F31/I57 were reduced to 15% and 40% compared with I31/V57 *in vivo* and *in vitro*. We considered the differences between the kinase activities and divided individuals into four categories of Aurora-A haplotype combination. Category I had 57.5% or less kinase activity compared with the most common category, category III, and had a significantly higher estimated cancer risk (odds ratio, 5.5328; 95% confidence interval, 1.8149–16.8671). Abnormal nuclear morphology, a characteristic of genomic instability, was observed to be 30 to 40 times more frequent in human immortalized fibroblast cells over-expressing I31/I57 or F31/I57 compared with the others. Furthermore, significantly higher levels of chromosomal instability were observed in cancers in category I (homozygote 91T-169A) than those in category III (homozygous 91A-169G). These results indicate that the less kinase active Aurora-A haplotype combinations might induce genomic instability and increase esophageal cancer risk either in a recessive or a dominant manner.

## Introduction

The majority of cancer susceptibility genes identified thus far have high penetrance, and germline abnormalities of the high-penetrance genes have been found in only a small proportion of cancer patients (1). In contrast, genetic susceptibility to common, sporadic cancer is caused by abrogation of multiple genetic loci of low-penetrance genes (2). Experimental animal studies have been used successfully to identify genes responsible for complex genetic traits, including cancer susceptibility (3,4). *Stk6* was identified as a low-penetrance cancer

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susceptibility gene responsible for the development of mouse skin tumors (5). The human orthologue of mouse *Stk6* gene, *Aurora2/STK15*, was also considered to be a human cancer susceptibility gene in population studies (6–8). Here we report on human *STK15*, commonly referred to as Aurora-A (9). The Aurora-A gene is located in 20q13, which is a chromosomal region that is frequently amplified in human tumors, and this gene is overexpressed in various human cancers, such as colorectal, breast, and bladder cancer (10–12). Aurora-A is a mitotic kinase required for the formation of a bipolar mitotic spindle and for accurate chromosome segregation in mammalian cells (13,14). Correlations between Aurora-A overexpression and aneuploidy have been reported in breast and gastric cancer (12,15).

The identification of subtle single nucleotide polymorphisms that affect gene function or expression and contribute to disease predisposition has become a major area of investigation towards understanding the mechanisms for complex diseases such as cancer (16). Two coding single nucleotide polymorphisms of human Aurora-A, 91T>A and 169G>A, have been reported in the Japanese single nucleotide polymorphism (17) and the Celera databases (18). Two coding single nucleotide polymorphisms in Aurora-A create four haplotypes, 91T-169G (T-G), 91A-169G (A-G), 91T-169A (T-A), and 91A-169A (A-A). The 91T>A polymorphism results in phenylalanine/isoleucine (F/I) at amino acid 31, and the other 169G>A polymorphism results in valine/isoleucine (V/I) at amino acid 57. These two polymorphisms fall within motifs in the $NH_2$-terminal region of Aurora-A that are termed Aurora Box1 (aa 5-40) and Aurora Box2 (aa 43-63). Both motifs are thought to play roles in ubiquitin-based proteolysis (19,20). Colon cancers from patients with the 91A allele encoding the 31I variant had significantly higher chromosomal instability measured by comparative genomic hybridization bacterial artificial chromosome (BAC) array analysis (5). The case-control study suggested that this polymorphism is also associated with esophageal squamous cell carcinomas (ESC; ref. 6). The amino acid sequence surrounding 57, [$^{56}$R(V/I)PL], contains a consensus [RxxL] sequence found in four amino acid stretches of Aurora-A, [$^{205}$RVYL], [$^{286}$RTTL], [$^{371}$RPML], or [$^{375}$REVL], in the kinase domain. Recently, *LATS2/kpm* has been identified as a specific substrate of Aurora-A kinase (21). *LATS2* is a mammalian homologue of the *Drosophila* tumor suppressor gene and encodes a serine/threonine kinase (22,23). In HeLa cells, overexpression of LATS2 causes $G_2$-M arrest through inhibition of Cdc2 kinase activity and also serves to induce apoptosis (24,25). Aurora-A colocalizes with LATS2 and phophorylates LATS2 at the 83rd serine residue (Ser83) in mammalian cells (21). The phosphorylation of Ser83 by Aurora-A may be important in suppressing tumor development and prohibiting cell proliferation. Somatic mutations of Aurora-A[5] and frequent allelic losses of 13q11–12, the locus for *LATS2/kpm* in the genome, are found in ESC (25). These results suggest that dysfunctions of Aurora-A or LATS2 may be involved in the development of esophageal cancer.

Thus, we hypothesized that the polymorphisms confer esophageal cancer risks as a consequence of their functional differences of Aurora-A isoforms. In this study, an E SC case-control study was done on the STK15 polymorphisms. Although there was no significant difference in the each genotype, subsequent functional analysis allowed us to identify that the kinase activity level of I31/I57, F31/I57, and F31/V57 variants was reduced to 15%, 40%, and 95%, respectively, compared with a common variant, I31/V57, *in vivo* and *in vitro*. We therefore analyzed and identified the association between ESC cancer risk and haplotype combinations that confer lower kinase activity. In this report we also observed significantly higher chromosomal instability in ESCs obtained from patients with a T-A (F31/I57) homozygous haplotype as compared with those from patients with the A-G (I31/V57) homozygous haplotype. Our findings have suggested that higher cancer risk of ESC in the

[5]M.T. Kimura, in preparation.

population may be explained by a further increase in chromosomal instability in somatic tumors developed from a germline background of the STK15 lower kinase activity.

## Materials and Methods

### Genotyping and haplotyping

In total, 197 esophageal squamous cancer cases from unrelated esophageal cancer patients and 146 controls from outpatients with either healthy status or benign disease were recruited from Tohoku University, Sendai, Japan. Ethnicity (Japanese), frequency of gender, and average age of the 146 controls were group matched to cases. DNA was extracted from blood samples by QIAamp Blood DNA Mini Kit (Qiagen, Hilden, Germany) and used as templates for the Aurora-A target region amplification by PCR with 5′-CATGAATGCCAGAAAGTTT-3′ forward primer and 5′-biotin-conjugated 5′-CTGATTCTGAACCGGCTTGT-3′ reverse primer. The PCR reaction was carried out in a 50-μL volume containing 40 ng of extracted DNA, 2.5 pmol of each primer, 250 μmol/L of each deoxynucleoside triphosphate, 1.0 unit of EX-TaqDNA polymerase (Takara Bio, Otsu, Japan), 10 mmol/L Tris (pH 8.3), 50 mmol/L KCl, and 1.5 mmol/L $MgCl_2$. Thermal cycling was done with an initial denaturation for 2 minutes at 95°C, succeeded by 35 cycles of denaturation for 20 seconds at 94°C, primer annealing for 20 seconds at 57°C, and synthesis for 20 seconds at 72°C. A final primer extension was conducted for 5 minutes at 72°C. All amplification reactions were done in a T1 Thermocycler (Biometra, Goettingen, Germany). Biotinylated amplified DNA fragments were attached to streptavidin sepharose (Amersham Biosciences, Buckinghamshire, United Kingdom), and purified single strand DNA templates were prepared as mentioned in a previous report (26) and analyzed on a PSQ96 instrument (Pyrosequencing AB, Uppsala, Sweden) with sequencing primers 5′-GTG-TTCTCGTGACTCAGCAA-3′ for the 91T>A genotyping and 5′-CTT-CAAATTCTTCCCAGCGC-3′ for the 169G>A genotyping. DNA sequence reading was conducted in a PSQ96 instrument (Pyrosequencing). To reveal the haplotype of the 91T/A-169G/A heterozygote samples, the PCR products were cloned into pGEM vector (Promega, Madison, WI), and the cloned DNA sequences were fully determined by the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). This study is approved by the Ethics Committee of Tohoku University and Roswell Park Cancer Institute Institutional Review Board.

### Statistical analysis

Deviation from Hardy-Weinberg equilibrium in controls and cases were assessed by $\chi^2$ analysis. Tests of the allele frequencies in controls indicated departures from Hardy-Weinberg equilibrium for both cases and control at 169 polymorphism (case $P = 0.023$ and control $P = 0.020$). Risk was estimated with odds ratios (OR) for esophageal cancer and corresponding 95% confidence levels (95% CI). OR and 95% CI of each genotype were calculated and compared with that of a common genotype as a reference group.

### Cell cultures

Immortalized human fibroblast cell line, MRC5-SV1TG1, was obtained from RIKEN Cell Bank. Primary dermal fibroblast cells were established from three Japanese men. These cells were maintained in DMEM (Invitrogen, Carlsbad, CA) with 10% FCS (Sigma, St. Louis, MO), 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C under humidified 5% $CO_2$.

### Plasmids

RNA was extracted using Trizol reagent (Invitrogen) according to the protocol of the manufacturer from the primary human fibroblast cells and then used for reverse transcription-PCR ( RT-PCR) to clone cDNA encoding F31/V57, I31/V57, F31/I57, or I31/I57 Aurora-A

isoform. cDNA was prepared using Superscript First-Strand Synthesis System for RT-PCR kit (Invitrogen) and the cDNA was amplified with forward primer 5′-GCGGATCCATGGACCGATCTAAAGAAAC-3′ and reverse primer with stop codon 5′-CCGCTCGAGCTAAGACTGTTTGC-TAGCTG-3′ or reverse primer without stop codon 5′-CCGCTCGAGGTAA-GACTGTTTGCTAGCTG-3′ with the following conditions: an initial denaturation for 2 minutes at 95°C, succeeded by 30 cycles of denaturation for 30 seconds at 94°C, primer annealing for 30 seconds at 54°C, and synthesis for 1 minute at 72°C, and a final primer extension for 5 minutes at 72°C. All amplification reactions were done in a T1 Thermocycler (Biometra). The RT-PCR products with the stop codon were digested with *Bam*HI and *Xho*I, cloned into pGEX4T-1 (Amersham Biosciences) for expression of $NH_2$-terminal glutathione *S* -transferase (GST)-fusion protein in bacteria cells, and the products without the stop codon were cloned into pLenti6 (Invitrogen) for expression of COOH-terminal V5-tagged ($NH_2$- GKPIPNPLLGLDST-COOH) protein in mammalian cells.

### *In vitro* kinase assays

The GST-Aurora-A proteins were produced in BL21(DE3)pLysS bacteria cells (Invitrogen) and purified with Glutathione Sepharose 4B (Amersham Biosciences). *In vitro* kinase assays were done with 1 μg of the purified GST-Aurora-A protein and 2 μg of substrate protein, myelin basic protein (Upstate Biotechnology, Inc., Lake Placid, NY) or GST-LATS2 (aa 79–257; Cyclex, Inc., Japan), in kinase buffer [20 mmol/L HEPES-KOH (pH 7.5), 5 mmol/L $MgCl_2$, 5 mmol/L $MnCl_2$, 1 mmol/L DTT, 8 μg/mL bovine serum albumin] containing 10 μCi [$\gamma$-$^{32}$P] ATP or 20 μmol/L ATP. The reaction mixtures were incubated at 30°C for 30 minutes and subjected to SDS-PAGE. $^{32}$P-labeled proteins were visualized by autoradiography, and phosphorylated LATS2 was detected by Western blot analysis using anti-phospho-Ser83-LATS2 antibody (Cyclex).

### Establishment of cells exogenously expressing Aurora-A or LacZ

For generation of lentivirus expressing Aurora-A or LacZ protein, plasmid pLenti6 constructs containing Aurora-A cDNA or lacZ cDNA were cotransfected to 293FT cells with pLP1, pLP2, and pLP/VSVG as described in the instruction manual of the manufacturer (Invitrogen). Forty-eight hours after transfection, each virus-containing medium was harvested and applied to MRC5-SV1TG1 cells with 6 μg/mL Polybrane (Sigma) for 12 hours. Cells expressing exogenous Aurora-A or LacZ were selected by culture with the growth medium containing 5 μg/mL Blasticidin S (Invitrogen).

### *In vivo* kinase assays

MRC5-SV1TG1 cells that stably expressed each V5-tagged Aurora-A isoform or LacZ protein were washed with PBS and lysed in lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L NaF, 1 mmol/L $Na_3VO_4$, 1 mmol/L phenylmethylsulfonyl fluoride, 1× protease inhibitor cocktail (Calbiochem, San Diego, CA)]. After centrifugation at 13,200 × *g* for 30 minutes, the clear lysates were immunoprecipitated with 0.5 μg of anti-V5 antibody (Invitrogen) coupled with sepharose (Amersham Biosciences) for 3 hours at 4°C. The immunocomplexes were washed and incubated with GST-LATS2 in the presence of 10 μCi [$\gamma$-$^{32}$P]ATP, and phosphorylated LATS2 was detected by autoradiography. Intensities of bands in the digitized image of the autoradiograph were analyzed by NIH image software, which can be downloaded from web site http://rsb.info.nih.gov/nihimage/Default.html.

### Immunofluorescence

Cultured mammalian cells were fixed in 4% paraformaldehyde for 30 minutes, permeated with 0.1% Triton X-100 in PBS for 2 minutes, and blocked with 1% bovine serum albumin in PBS for 30 minutes at room temperature. The samples were incubated with 2 μg/mL anti-V5

antibody conjugated to FITC (anti-V5-FITC; Invitrogen) or with 1 μg/mL anti-phospho-Ser10-histone H 3 (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour. After washing samples with PBS for 30 minutes, secondary antibody coupled with Alexa Fluor-594 (Molecular Probes, Eugene, OR) was used for detection of phospho-histone H3, and samples were washed with PBS for 30 minutes. DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA).

### Array-based comparative genomic hybridization analysis

Genomic DNA samples were prepared from esophageal tumor tissues diagnosed as squamous cell carcinomas using DNeasy Tissue Kit (Qiagen). A total of 20 samples were examined with 10 possessing the 91-169 A-G/A-G haplotype and the other 10 with the 91-169 T-A/T-A haplotype. All the ESC samples were collected from male (58–78 years old) patients diagnosed as squamous cell carcinoma. One microgram of control and tumor DNA was labeled with fluorolink Cy3-dUTP or Cy5-dUTP (Perkin-Elmer, Wellesley, MA) using a BioPrime DNA labeling system (Invitrogen). Unincorporated fluorescent nucleotides were removed by Qiagen spin column (Qiagen). Prepared probes were hybridized with arrays consisting of 6,120 BAC clones from the RPCI-11 human genomic DNA BAC library as described previously (27). Each BAC clone was spotted in duplicate at 280-mm intervals on the arrays. Lists of BAC clones used in this study are shown at http://genomics.roswellpark.org. The array-based comparative genomic hybridization analyses were done as a sex mismatch to provide an internal hybridization control for chromosome X and Y copy number differences. The hybridized slides were scanned using a GenePix 4200A scanner (Axon, Inc.) to generate 10-μm resolution images for both Cy3 and Cy5 channels. Image analysis was done using ImaGene V4.1 (BioDiscovery, El Segundo, CA). A log 2 transformation was applied to the Cy5 (tumor) and Cy3 (control) background corrected means and these values were LOESS (Local Polynomial Regression Fitting) corrected to account for nonlinearities (28,29). For BACs where at least one of the replicate spots passes standard quality control steps, the mean of the log 2 tumor/normal values was calculated. Mapping information was added to the resulting log 2 tumor/normal values by querying the Human Genome Browser (May 2004 build) http://genome.ucsc.edu.

## Results

### Cancer risk and genotype or haplotype of Aurora-A

We genotyped DNA samples extracted from peripheral blood of 197 human ESC cases and 146 controls on the 91T>A and 169G>A polymorphisms by the Pyrosequencing method (26). The allele frequency of the 91T>A had ethnic diversity [White American: T/A = 0.7734:0.2265 ($n$ = 520), and the Japanese control: T/A = 0.3630:0.6370 ($n$ = 292)] and raised a problem of population stratification, whereas those of the 169G>A had no ethnic diversity [White American: G/A = 0.82:0.18, and the Japanese control: G/A = 0.8390:0.1610 ($n$ = 292)]. The allele frequency of 169G>A has been reported previously as G/A = 0.8585:0.1415 ($n$ = 1478) in an unrelated Japanese general population (17), which is similar to our genotyping study results, and confirming our reasoning for this study. The genotype A/A homozygosity at the nucleotide position 169 (169A/A) was present in 7.1% of esophageal cancer cases but absent in the controls [$\chi^2$ 2 degrees of freedom ($df$, 15,14), $P$ = 0.001]. The frequency of 169G/G was 59.9% in the cases and 67.8% in the controls, and that of 169G/A was 33.0% in the cases and 32.2% in the controls. The OR calculated from combining two genotypes of 169G/A and 169A/A was 1.4102 [95% confidence interval (95% CI), 0.9000–2.2097; Table 1]. There is no clear association between each of the genotypes and cancer risk. In a haplotype analysis of T-G, A-G, T-A, and A-A, the ORs for the A-A (OR, 3.1452; 95% CI, 1.0258-9.6435; $P$ = 0.04) and the T-A (OR, 1.5214; 95% CI, 0.9984–2.3183; $P$ = 0.05) were elevated (Table 2).

We have initiated functional analysis of each of the four Aurora-A isoforms based on these results.

## Kinase activity of Aurora-A isoforms

Functional differences among the Aurora-A isoforms, F31/V57, I31/V57, F31/I57, and I31/I57 proteins, were investigated to identify evidence supporting the above observation in case and control studies. First, the intrinsic kinase activity of the isoforms was investigated by *in vitro* kinase assays using myelin basic protein as a substrate in the presence of [$\gamma$-$^{32}$P]ATP. Recombinant F31/V57 and I31/V57 Aurora-A proteins showed significantly higher kinase a ctivity compared with F31/I57, and I31/I57 showed the weakest activity among the four isoforms (Fig. 1*A*). The human *LATS2* gene, a homologue of the *drosophila* tumor suppressor protein, interacts with Aurora-A, the Ser83 of LATS2 is phosphorylated by Aurora-A, a nd LATS2 and Aurora-A colocalize at the centrosome (21). LATS2 is thought to be a phosphorylation target of Aurora-A in the native condition. We therefore did the same *in vitro* kinase assays with LATS2 as a substrate, and the results were similar to those against myelin basic protein (Fig. 1*A*). The Ser83 residue of LATS2 is phosphorylated by Aurora-A (21). We did Western blot analysis with anti-phospho-Ser83-LATS2–specific monoclonal antibody. F31/V57 and I31/V57 Aurora-A isoforms again showed higher phosphorylation activity of this residue than F31/I57, and I31/I57 had no activity (Fig. 1*B*). To quantify the degree of LATS2 Ser83 phosphorylation, an ELISA was done using an Aurora-A Assay/Drug Discovery Kit (Cyclex). The enzymatic activities of F31/V57 and I31/V57 were significantly higher than those of F31/I57 and I31/I57 (Fig. 1*C*). These ELISA results were consistent with the kinase assay using the isotope in Fig. 1*A* and with Western blot results in Fig. 1*B*. We prepared immunocomplexes from immortalized human MRC5-SV1TG1 fibroblast cells that express each isoform of Aurora-A with V5-tag ($NH_2$-GKPIPNPLLGLDST-COOH) to investigate the kinase activity of the four isoforms *in vivo*. We measured $^{32}$P-labeled Lats2 signal using an *in vivo* kinase assay (Fig. 1*D*). The results showed that the relative intensity by I31/I57, F31/I57, or F31/V57 was 22.9 ± 4.4%, 59.2 ± 9.3%, or 97 ± 4.2%, respectively, compared with that of I31/V57. I31/I57 showed the weakest activity also *in vivo* (Fig. 1*D*). Therefore, the risk haplotypes, I31/I57 and F31/I57, have lower kinase activity against the LATS2 protein *in vitro* and *in vivo*. We estimated relative kinase activity from the *in vitro* and *in vivo* kinase assays for I31/I57, F31/I57, F31/V57, and I31/V57 as 0.15, 0.40, 0.95, and 1.00, respectively.

## Cancer risk and haplotype combination of Aurora-A

We characterized haplotype combination categories by estimating total kinase activity in cells as a sum of the kinase activity calculated from the results of *in vivo* and *in vitro* kinase assays. As seen in Table 3, four distinct categories can be considered, and statistical analysis was done on each category. A category showing estimated kinase activity of 115 (57.5%) or less was classified as category I. The combined OR of category I was 5.5328 (95% CI, 1.8149–16.8671; $P < 0.001$), and it showed significant risk compared with A-G/A-G, category III, which has an estimated kinase activity of 100%. ORs of category II and category IV showing estimated kinase activities of 67.5–70% and 90–97.5%, respectively, suggested that the cancer risk was similar to that of category III. These results suggest that T-A (F31/I57 variant form) functions may confer susceptibility to esophageal cancer in a recessive manner, whereas A-A (I31/I57 variant form) may do so in a dominant manner.

## Subcellular localization of Aurora-A isoforms

We then examined the localization of the four isoforms of Aurora-A. Localization of endogenous Aurora-A protein is regulated in a cell cycle–dependent manner. Aurora-A begins to localize to the centrosomes only in S phase, soon after the centrioles have been duplicated.

The protein is then degraded in early $G_2$-M (12,30). We established MRC5-SV1TG1 cells expressing each of the four isoforms of Aurora-A protein with a V5 tag and observed the localization of exogenously expressed Aurora-A isoform using anti-V5 antibody (Fig. 2*A*–*L*). All four types of Aurora-A proteins were condensed in the centrosomes during metaphase (Fig. 2*G*). During interphase, exogenously expressed low-risk F31/V57 or I31/V57 isoform was homogeneously localized only in the nuclei (Fig. 2*M*). Risk-type Aurora-A isoforms (I31/I57 and F31/I57) showed significant increases in percentage of abnormal diffuse localization in the cytosol in addition to the nuclear localization (Fig. 2*N* and *O*).

### Nuclear morphological changes induced by exogenous expression of Aurora-A

Nuclear morphologic abnormalities in Aurora-A stable clones were examined by DAPI staining. Expression of all four isoforms resulted in changes in nuclear morphology compared with control cells expressing the LacZ protein. Representative morphologic changes of the nuclei in the cells exogenously expressing I31/I57 are shown in Fig. 3*A*. Frequency of abnormal nuclei induced by exogenous F31/V57, I31/V57, F31/I57, and I31/I57 expression was respectively 11.2 ± 1.1, 7.2 ± 2.1, 32.9 ± 2.3, and 39.9 ± 4.5 times higher than that of control cells expressing the LacZ protein (Fig. 3*B*). Risk-type isoforms, F31/I57 and I31/I57, produced abnormal nuclei about three to five times more frequently than non-risk types, F31/V57 and I31/V57.

### DNA copy number aberrations and Aurora-A haplotype combination

We carried out BAC array–based comparative genomic hybridization analysis of ESC genomes containing homozygote haplotype combination A-G/A-G or homozygote T-A/T-A. Ten ESC samples with each haplotype combination were analyzed by array-based comparative genomic hybridization to generate log 2 ratio profiles of each BAC clone. We analyzed 4,612 ± 125 BAC data points covering the autosomes per sample from A-G/A-G haplotype combination and 4,539 ± 296 from T-A/T-A combination. We counted the number of BAC spots of which log 2 ratios were more than 0.37 (absolute ratio 1.292) or less than −0.5 (absolute ratio 0.707) to calculate the number of genomic loci showing aberrant copy numbers. Frequency of chromosomal loci with aberrant copy number changes in the T-A/T-A haplotype combination (FI/FI) was higher than that in the A-G/A-G combination (IV/IV) on all autosomes (Fig. 3*C*). The aberrant frequencies of each 20 ESC genomes were calculated. The average of the frequency of the T-A/T-A haplotype combination was 0.6 ± 0.7% and that of the A-G/A-G combination was 6.7 ± 4.4% (Fig. 3*D*).

## Discussion

A simple association study analyzing the genotypes of the two Aurora-A coding single nucleotide polymorphisms did not show a significant association with cancer risk, although a previous report indicates that the 91T>A polymorphism site is associated with cancer risk of ESC (6). Our data indicated 169G>A polymorphism also affects esophageal cancer risk. This difference may be explained by either the differences of population used for the case-control studies or complicated mechanisms that increase cancer risk associated with Aurora-A. The haplotype analysis of the two single nucleotide polymorphisms showed the OR of A-A (OR, 3.1452; 95% CI, 1.0258–9.6435) or T-A (OR, 1.5214; 95% CI, 0.9984–2.3183) compared with A-G. This data suggested the isoforms encoded in these alleles had a tendency to increase cancer risk and might have functional difference(s). Frequency of the A-A allele is ~3% in Japanese and 1% in the caucasian American population, and that of the T-A allele was around 30% in both Japanese and caucasian American populations. When we analyzed functions of the four Aurora-A isoforms, I31/I57 encoded in the A-A allele or F31/I57 encoded in T-A showed 15% or 40% of kinase activity, compared with I31/V57 variants, although both isoleucine and valine have nonpolar side chains. This result suggested that the 57th amino acid

residue is important for its kinase activity. Furthermore, we estimated the association between haplotype (allele) combination and cancer risk from the esophageal cancer case and group-matched control study. We categorized haplotype combinations of Aurora-A alleles into four categories, according to the estimated total kinase activity, and determined that haplotype combination category I showed a significant association with esophageal cancer risk. The result also suggests that I31/I57 elevates cancer risk in a dominant manner and F31/I57 elevates cancer risk in a recessive manner. Dose-dependent reduction of the kinase activity may therefore cause the increased esophageal cancer risk.

In addition to the difference of the kinase activity among the isoforms, I31/I57 and F31/I57 localized not only in the nuclei but also frequently in the cytosol, although the other isoforms localize only in the nuclei. Abnormal subcellular or subnuclear localization of Aurora-A might induce missegregation of chromosomes during mitosis, resulting in the observed abnormal nuclear shape. Nuclear morphologic changes were more frequently induced in cells overexpressing the I31/I57 Aurora-A isoform than in cells over-expressing the other isoforms (Fig. 3*B*). Similar nuclear morphologic changes are induced by suppressing Aurora-B protein level using RNA interference (31). Quantitative or qualitative imbalance of Aurora proteins has been shown to cause abnormal chromosome segregation and aneuploidy (12,15). Genomic instability of ESCs with the T-A/T-A haplotype combination, which is categorized into the high-risk category I, was about 10-fold higher than that with the IV/IV combination, non-risk category III (Fig. 3*D*). Aurora-A risk isoforms might increase cancer susceptibility by inducing abnormal centrosome segregation and imbalanced chromosome segregation due to either their kinase deficiencies in the phosphorylation of a substrate, LATS2, and/or their inappropriate cellular localizations. Although further analysis is necessary to determine whether these risk haplotypes may also cause cancer development at higher frequency in other populations, our finding of functionally different isoforms has profound implications for this possibility.

Aurora-A amplification is frequently observed in human cancers, such as breast, colon, and ovarian carcinoma. Thus, Aurora-represents a therapeutic target molecule, and kinase inhibitors against Aurora-A have been identified as potential therapeutic agents for cancer patients (32). Patients with low kinase activity of Aurora-A could occur with greater frequency in the high-risk category than in the low-risk population. The kinase activity of cancer patients may influence the effectiveness of clinical response to therapy using such kinase inhibitors. The Aurora-A polymorphisms need to be considered as a modification factor for cancer treatments using such specific kinase inhibitors and for subsequent clinical trials. This study has a lso raised a possibility of long-term side effect of Aurora kinase inhibitors due to the elevated chromosomal instability.

Abnormally high Aurora-A kinase activity has been thought of as one of the necessary steps for cancer developments, however, our results indicate that the population with the lower-kinase activity type Aurora-A isoforms has a higher cancer risk. It might be that an abnormally high Aurora-A kinase activity is required for progression of cancer cells but not for initiation of tumors, or that an inappropriate level of Aurora-A kinase activity level, either excess or reduced kinase level, may increase cancer risk. Further study is required to better understand the mechanisms how risk-type Aurora-A isoforms trigger development of cancer.

We found that cancer risk was significantly increased in individuals with a haplotype combination category I. Individuals within this category make up 2% to 3% of the population and may have a 5-fold increase in cancer risk. Genetic testing of Aurora-A polymorphisms or Aurora-A kinase assays may be useful for detecting high-risk individuals, and the results may encourage the higher-risk population to have frequent medical examinations to detect early-stage cancer.

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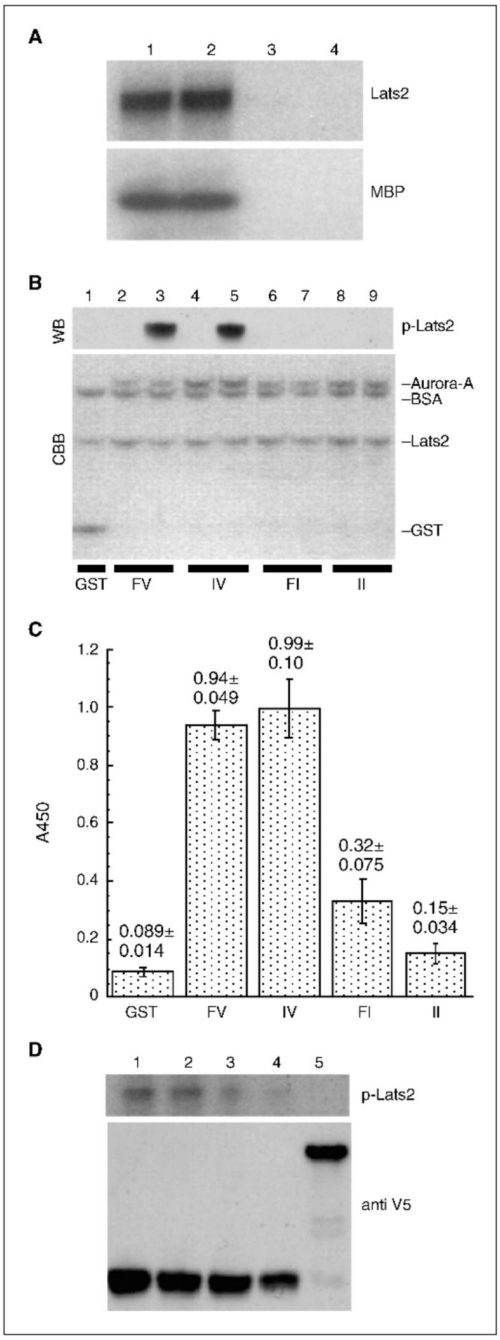


**Figure 1.**

*A*, Aurora-A kinase activity for the LATS2 Ser83. Each of the four Aurora-A isoforms, F31/V57 (*lane 1*), I31/V57 (*lane 2*), F31/I57 (*lane 3*), and I31/I57 (*lane 4*), was used for *in vitro* kinase assay using myelin basic protein (Upstate Biotechnology) or recombinant LATS2 protein (aa 79–273) as substrates. *B*, LATS2 phosphorylation was confirmed by Western blots using anti-phospho-LATS2 (Ser83) antibody. GST (*lane 1*), Aurora-A protein isoforms F31/V57 (*lanes 2–3*), I31/V57 (*lanes 4–5*), F31/I57 (*lanes 6–7*), and I31/I57 (*lanes 8–9*) were incubated with recombinant LATS2 protein (aa 79–273) in the presence (+) or absence (−) of ATP. Representative results of Western blot (*top*) and of Coomassie blue staining (*bottom*). *C*, quantitative analysis for kinase activity of each Aurora-A isoform to LATS2 by ELISA

using the Aurora2 Assay kit (Cyclex). Activity of each GST-Aurora-A isoform. The assays were repeated in triplicate; *columns*, mean; *bars*, SD. D, *in vivo* Aurora-A kinase activity against LATS2. Kinase activity of the immunocomplexes containing F31/V57 (*lane 1*), I31/V57 (*lane 2*), F31/I57 (*lane 3*), I31/I57 (*lane 4*), and control LacZ (*lane 5*) were visualized by autoradiography (*top*), and amount of Aurora-A isoforms or LacZ protein in the immunocomplexes was confirmed by Western blot (*bottom*). Relative intensities of I31/V57 (*lane 2*), F31/I57 (*lane 3*), and I31/I57 (*lane 4*) compared with F31/V57 (*lane 1*) were 97 ± 4.2%, 59.2 ± 9.3%, and 22.9 ± 4.4%, respectively.

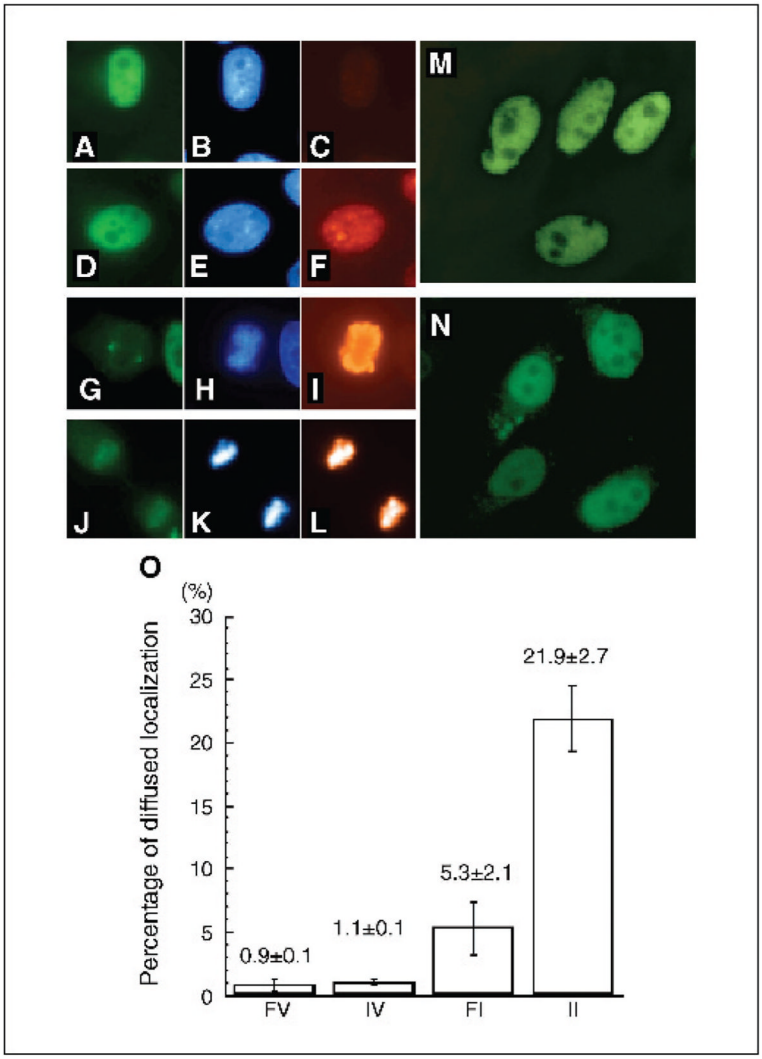


**Figure 2.**

Localization of exogenous V5-tagged F31/V57 Aurora-A protein during different phases of cell cycle: *A–C*, interphase; *D–F*, prophase; *G–I*, prometaphase; and *J–L*, meta-anaphase. The cells at each stage of cell cycle were stained for exogenously expressed Aurora-A protein (*A*, *D*, *G*, and *I*), DNA (*B*, *E*, *H*, and *K*), and Ser10 phosphorylated histone H3 (*C*, *F*, *I*, and *L*). Typical nuclear localization pattern of V5-tagged F31/V57 localization (*M*) or nuclear plus cytoplasmic localization patterns of V5-tagged I31/I57 (*N*) in MRC5-SV1TG1 cells are shown. *O*, frequencies of cells with the ectopic diffuse localization were calculated from a total count of 1,500 cells (500 each of three independent stable clones). *Columns*, mean of three clones in each isoform; *bars*, SD.

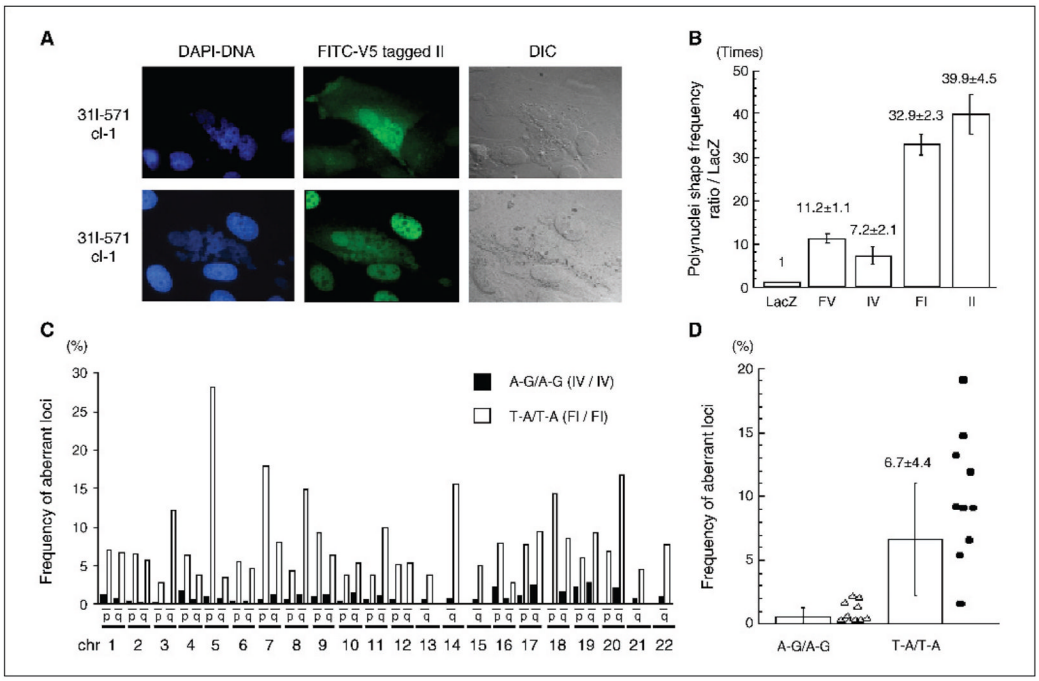


**Figure 3.**

*A*, representative abnormal nuclear shapes found in MRC5-SV1TG1 cells expressing I31/I57 Aurora-A. DAPI images for nuclei, FITC images for V5-tagged Aurora-A, and differential interference contrast (*DIC*) images. *B*, frequency of cells with abnormal nuclear shape is compared with those in LacZ control. *Columns*, mean of triplicate determinations; *bars*, SD. *C*, array-based comparative genomic hybridization analysis of genome copy number in esophageal tumor tissues. Percentages of aberrant loci, the comparative genomic hybridization log 2 ratios for each BAC clone more than 0.37 (absolute ratio > 1.292) or less than −0.5 (absolute ratio 0.707), are presented based on chromosome position. *Black bar*, results of the A-G/A-G haplotype combination (IV/IV); *white bar*, results of the T-A/T-A combination (FI/FI). *D*, genomewide frequencies of copy number aberrations of the each esophageal squamous cancer tissues are plotted. *Open triangles*, frequency of the each ESC with A-G/A-G haplotype combination (IV/IV); *closed circles*, frequency of the T-A/T-A combination (FI/FI). *Columns*, mean of the plotted value; *bars*, SD.

**Table 1**

Estimated risk of esophageal cancer and Aurora-A genotypes

| | Control (*n* = 146) | Case (*n* = 197) | OR (95% CI) | | OR (95% CI) |
|---|---|---|---|---|---|
| Genotype 91 | | | | | |
| T/T | 12 (8.2%) | 29 (14.7%) | 1.9333 (0.8994–4.1556) | ] | 1.1234 (0.716–1.7627) |
| T/A | 82 (56.2%) | 103 (52.3%) | 1.0049 (0.6306–1.6014) | | |
| A/A | 52 (35.6%) | 65 (33.0%) | 1 | | 1 |
| Genotype 169 | | | | | |
| G/G | 99 (67.8%) | 118 (59.9%) | 1 | | 1 |
| G/A | 47 (32.2%) | 65 (33.0%) | 1.1603 (0.732–1.8392) | ] | 1.4102 (0.9–2.2097) |
| A/A | 0 (0%) | 14 (7.1%) | undetermined | | |

NOTE: OR and 95% CI are calculated compared with 91A/A and 169G/G as reference genotypes.

**Table 2**

Estimated risk of esophageal cancer and Aurora-A haplotypes

| Haplotype | Control (*n* = 292) | Case (*n* = 394) | OR (95% CI) |
|---|---|---|---|
| 91T-169G (T-G) | 63 (21.6%) | 86 (21.8%) | 1.1183 (0.7636–1.6377) |
| 91A-169G (A-G) | 182 (62.3%) | 223 (56.6%) | 1 |
| 91T-169A (T-A) | 43 (14.7%) | 70 (17.8%) | 1.5214 (0.9984–2.3183) |
| 91A-169A (A-A) | 4 (1.4%) | 15 (3.8%) | 3.1452 (1.0258–9.6435) |

NOTE: OR and 95% CI are calculated compared with A-G as a reference haplotype.

**Table 3**

Estimated esophageal cancer risk of Aurora-A haplotype combinations

| Haplotype combination91-169/91-169 | Control, *n* = 146 (%) | Case, *n* = 197 (%) | OR (95% CI) | Total Aurora-A kinase activity* | Haplotype combination category† | OR of each category (95% CI)‡ |
|---|---|---|---|---|---|---|
| A-A/A-A | 0 (0) | 0 (0) | undetermined | 15.0 | I | 5.5328 (1.8149–16.8671) |
| A-A/T-A | 0 (0) | 2 (1.0) | undetermined | 27.5 | | |
| T-A/T-A | 0 (0) | 12 (6.1) | undetermined | 40.0 | | |
| A-A/A-G | 2 (1.4) | 9 (4.6) | 3.6885 (0.7619–17.8568) | 55.0 | | |
| A-A/T-G | 2 (1.4) | 4 (2.0) | 1.6393 (0.2883–9.322) | 57.5 | | |
| T-A/T-G | 7 (4.8) | 11 (5.6) | 1.2881 (0.4651–3.5677) | 67.5 | II | 0.9912 (0.5716–1.7188) |
| T-A/A-G | 36 (24.7) | 41 (20.8) | 0.9335 (0.521–1.6727) | 70.0 | | |
| A-G/A-G | 50 (34.2) | 61 (31.0) | 1 | 100.0 | III | 1 |
| T-G/T-G | 5 (3.4) | 6 (3.0) | 0.9836 (0.2834–3.4137) | 90.0 | IV | 0.9535 (0.5588–1.6271) |
| A-G/T-G | 44 (30.1) | 51 (25.9) | 0.9501 (0.5482–1.6467) | 97.5 | | |

*Kinase activity levels were estimated from the results in Fig. 1 (A-G = 1.00, T-G =0.95, T-A = 0.40, and A-A = 0.15, see text).

†The haplotype combinations were classified into four categories according to kinase activity.

‡The OR (95% CI) of each category was calculated using the A-G/A-G haplotype combination as a reference category.