Amplification and overexpression of *Aurora-A* in esophageal squamous cell carcinoma

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Abstract. *Aurora-A/BTAK/STK15* gene which encodes a centrosome-associated kinase is located on chromosome 20q13.2, a highly amplified region in various human tumors. Recent studies have demonstrated the overexpression and amplification of *Aurora-A* in many malignant human cancers. The purpose of this study was to investigate the amplification and expression of *Aurora-A* in esophageal squamous cell carcinoma. Amplification of *Aurora-A* was determined by fluorescence *in situ* hybridization in 7 esophageal cancer cell lines and real-time PCR in 29 esophageal cancer samples. We detected *Aurora-A* expression in 7 esophageal cancer cell lines and 38 esophageal cancers samples by semi-quantitative reverse transcription-PCR and Western blot hybridization. The amplification of *Aurora-A* was detected in 27 of 29 (93.1%) esophageal cancer samples and 6 of 7 (85.7%) cancer cell lines. *Aurora-A* was overexpressed in 27 of 38 (71.1%) esophageal cancer samples and all 7 esophageal cancer cell lines. We conclude that *Aurora-A* is amplified and overexpressed in esophageal squamous cancer.

Introduction

The centrosomes are important for cells to maintain genomic stability through establishment of bipolar spindles during cell division, which could allow equal segregation of replicated chromosome to split into two daughter cells. The centrosomal function is strictly regulated by various proteins. Aurora kinase family, a conserved mitotic serine/threonine kinase family, played a vital role in regulating the centrosomal and microtubule function, ensuring accurate chromosome segregation and efficient completion of cytokinesis (1,2). Among the three members of this family, Aurora-A, attracted most attention because *Aurora-A* gene is mapped to chromosome 20q13.2, a region amplified commonly in epithelial cancers (3-6).

Amplification and/or overexpression of *Aurora-A* is detected in several types of tumors, including gastric cancer, bladder cancer, gliomas and hepatocellular cancers (7-12). Combining with the studies that overexpression of *Aurora-A* can transform NIH3T3 and Rat1 cells (13,14) and increase the incidence of breast cancer in transgene mouse (15), most researchers would consider *Aurora-A* as an oncogene.

ESCC is the fourth most prevalent malignancy in China, but the molecular mechanism for the tumorigenesis of esophageal cancer remains to be elucidated. Gain of chromosome 20q was often observed in ESCC (16-20). There are no studies investigating DNA status of oncogene *Aurora-A* located on the 20q in ESCC despite recent reports showing the overexpression of *Aurora-A* in ESCC (12,21). Therefore, to further reveal the possible role of Aurora-A in esophageal cancer, we detected the DNA copies and expression level of *Aurora-A* in both esophageal cancer cells lines and primary tumor samples.

Materials and methods

Cell culture. Seven types of esophageal cancer cell lines KYSE150, 180, 410, 510, 70 (kindly provided by Professor Yutaka Shimada), ESCC109, ESCC9706 (kindly provided by Professor Mingrong Wang) and HLF (human lung fibroblast cell, kindly provided by Dr Youyong Lv, Peking University School of Oncology and Beijing Institute for Cancer Research, Beijing, P.R. China) were cultured in RPMI-1640 medium supplemented by 10% fetal bovine serum at 5% CO_2.

Clinical tissue sample collection. We obtained 38 esophageal squamous cell carcinoma tissues and corresponding normal tissues from surgically resected esophageal carcinoma at the Cancer Hospital. None of the patients investigated received

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chemotherapy or radiotherapy prior to the operation. All the samples were histopathologically diagnosed as esophageal squamous cell carcinoma.

Fresh samples were dissected manually to remove connective tissues and stored immediately at -80˚C until analyzed. The corresponding normal tissues were obtained from the distant edge of dissected esophagus without carcinoma cell invasion by pathological diagnosis. Every tissue sample contained over 80% of normal or tumor epithelial cells.

Fluorescence in situ hybridization (FISH). Cells were treated with 1 μg/ml colchicines overnight. Then the metaphase cell slides were made according to standard protocol.

Dual-color FISH was undertaken to detect 20q13.2 amplification in esophageal cancer cell lines. We co-hybridized an *Aurora-A* specific BAC probe (kindly provided by Dr Subrata Sen and Ms. Hongyi Zhou, University of Texas, M.D. Anderson Cancer Center) with a chromosome 20 specific centromere probe. The probes were labeled with biotin or digoxin by nick translation using Nick Translation Kit (Gibco/BRL, Rockville, MD, USA). Hybridization was performed at 37˚C for 48 h in a moist chamber. After hybridization, the slides were washed in 50% formamide/2X SSC three times for 5 min each, followed by washes in 2X SSC three times for 5 min each time. Then the slides were blocked by 4X SSCB (4X SSC with 0.5% Blocking Regent bought from Roche 0.02% NaN_3) at room temperature for 30 min. The slides were incubated with Avidin-FITC in wet chamber at 37˚C for 60 min followed by three washings at room temperature for 5 min in 4X SSC. Amplification of the signals was performed by biotinylated anti-avidin antibody (Vector Laboratories, Burlington, Canada). At 37˚C for 60 min in a moist chamber followed by three washings at room temperature with 5 min wash in 4X SSC. A final incubation with Avidin-FITC and Rhodamine-anti-digoxigenin (Roche, Mannheim, Germany) (working solution: 5 μg Avidin-FITC/ml in 4X SSC, 0.5% blocking reagent, 0.2% NaN_3) was done at 37˚C for 60 min in a moist chamber followed by three washings at room temperature for 5 min in 4X SSCB. The nuclei were counterstained with DAPI.

The 20q13.2 amplification was recognized by comparison of the numbers of red and green signals in cell nuclei. Approximately 100-200 cells were numbered for each type of cells when summarizing the 20q13.2 amplification rate.

RNA extraction and semi-quantitative RT-PCR. Total-RNA was extracted from paired specimen of primary esophageal cancer and non-cancerous esophageal epithelium with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Five microgram of total-RNA extracted from paired esophageal carcinoma was used as template respectively to synthesize cDNA in 25 μl reaction mixture with 2.5 mM oligo(dT) primers and M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) at 37˚C for 1 h followed by 85˚C for 10 min. PCR was performed in 20 μl reaction mixture [1X AmpliTaq buffer, 100 ng template cDNA, 200 μM of each dNTPs, 0.5 μM of each primers and 2 U AmpliTaq (Roche)] as follows: 95˚C for 5 min followed by 24 cycles of 95˚C for 1 min, 56˚C for 1 min, 72˚C for 1 min, and the final step of extension was for 10 min at 72˚C. Sequences of the PCR primers for *Aurora-A* are as follows: upper primer: 5'-GCCTCCTGTGAAGACACCAT-3'; lower primer: 5'-ACTT TGTAACAGAGGGAGCC-3' with an expected product of 471 bp. ß-actin was used as internal control (upper: 5'-GGCG GCACCACCATGTACCCT-3' and downstream: 5'-AGGGG CCGGACTCGTCATACT-3') at 202 bp. Negative control was the amplification omitting the template from the reaction. The reaction products were visualized by electrophoresis of 5 μl reaction mixture at 70 V for 40 min in 2% agarose gel containing 0.5 μg/ml ethidium bromide, and quantitated by densitometry using Gel-Pro Analyzer version 3.1 (Media Cybernetics, Silver Spring, MD, USA).

Real-time polymerase chain reaction. To detect the amplification in cancer samples, real-time PCR was performed in the Rotor-Gene RG-3000 Real-Time Thermal Cycler (Corbett Research, Sydney, Australia), which detects the signal from the fluorogenic probe during PCR. As both the precise amount of genomic DNA and its quality are difficult to assess, we also quantified a control gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). All data were normalized to *GAPDH*.

The ratio defining the level of amplification is termed 'Am', and is determined as follows:

$$Am = \frac{T_{\text{the ratio of gene copy number of } \textit{Aurora-A} \text{ to } \textit{GAPDH}}}{N_{\text{the ratio of gene copy number of } \textit{Aurora-A} \text{ to } \textit{GAPDH}}}$$

(T, tumor tissues; N, corresponding normal tissues).

The kinetic method requires a standard curve which was constructed with serial 10-fold dilutions of specific PCR products. And in this study, we constructed two standard curves with two different pairs of primers used later for real-time quantitative PCR to quantify *Aurora-A* and *GAPDH* gene copy number separately.

PCR amplifications were done with SYBR Premix Ex Taq (Perfect Real-Time) Kit (Takara Biotechnology, Dalian, P.R. China) according to the manufacturer's protocol. The amplification mixes (25 μl) contains SYBR Premix Ex Taq 12.5 μl, ROX Reference Dye 0.5 μl, each primer 0.5 μl (200 nM) and template 2 μl (around 20 ng). The fragment of *Aurora-A* was amplified with the primers 5'-CCTTCGAATGTTGGCAGG AT-3' and 5'-TCCAACACTAACAGACCGCA-3'. Internal control *GAPDH* used the primers 5'-TGAAGGTCGGAGTC AACGGA-3' and 5'-CATGTGGGCCATGAGGTCCA-3'. PCR was performed as follows: 94˚C for 10 sec followed with 40 cycles of 94˚C for 5 sec and 60˚C for 30 sec. The software Rotor-Gene 5.0 calculated the parameter C_t (threshold cycle) and determined the starting copy number in the samples.

We investigated 29 cancer samples and their corresponding normal tissues. The ratio of *Aurora-A* copy number to the copy number of *GAPDH* indicated the gene copy number level of *Aurora-A*.

Western blot analyses. In order to determine the expression levels of Aurora-A in esophageal cancer cell lines and cancer samples, the frozen tissues after trituration and culture cells

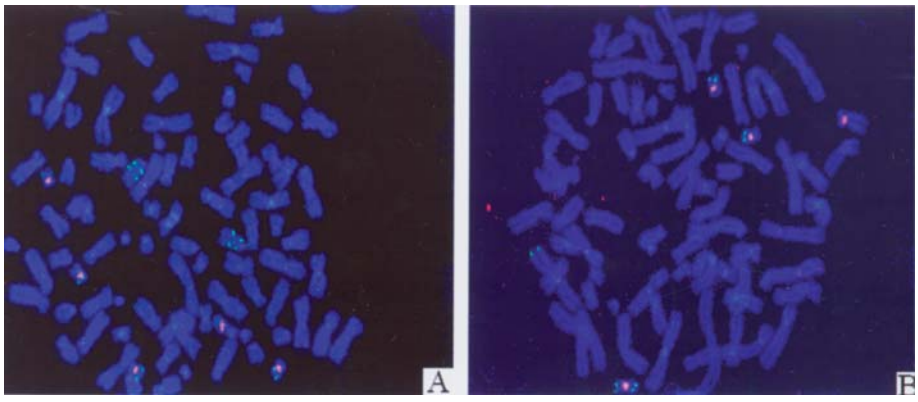

Figure 1. Amplification of *Aurora-A* in esophageal cancer cell lines. Fluorescence *in situ* hybridization was used to detect the amplification of *Aurora-A* in metaphase cells by using *Aurora-A* gene specific probe (green signal) and centromere probe for chromosome 20 (red signal). There are more green signals than red signals in esophageal cancer cells KYSE410 (A) and KYSE510 (B).

Table I. Amplification of *Aurora-A* in esophageal cancer cell lines.

Esophageal cancer cell lines	Numbered cell	Ratio[a]		Amplification rate (%)	High amplification (%)
		1-1.5	≥1.5		
KYSE150	113	6	93	87.6	82.3
KYSE410	144	18	125	99.3	86.8
KYSE510	152	121	3	81.6	1.97
KYSE180	131	24	107	100	81.7
KYSE70	172	4	0	2.32	0
ESCC109	106	93	2	89.6	1.89
ESCC9706	144	82	11	64.6	7.64

[a]Ratio between 20q13.2 and 20q centromere signal number in each cell line. For each cell type, 100-200 cells were counted.

were lysed in RIPA lysis buffer containing 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 μg/ml aprotinin and 5 μg/ml leupeptin. Lysates were centrifuged at 12,000 g for 15 min at 4˚C. The protein concentration was determined using BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Equal amounts of total proteins were then separated on 12% acrylamide gels using standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) techniques. After transfer to nitrocellulose membranes (Optitran, Schleicher&Schuell, Keene N.H., USA), the proteins were detected with anti-Aurora-A antibody (N20; 1:1000; Santa Cruz, CA, USA), followed by anti-goat IgG-horseradish peroxide-conjugated secondary antibody (Zhongshan Co., Beijing, P.R. China) and chemiluminescence luminol detection kit (Santa Cruz). The same membrane was reprobed with anti-ß-actin (AC-15; 1:5000; Sigma, St Louis, MO, USA) as a control for equivalent protein loading.

Results

Amplification of Aurora-A in esophageal cancer cell lines. Using an *Aurora-A* gene specific probe and centromere probe for chromosome 20, we detected the *Aurora-A* gene copy increments in esophageal cancer cell lines by FISH (Fig. 1). Each numbering included 100-200 metaphase cells, and total amplification rate in each cell type was calculated. Then the cells were assorted into amplification and high amplification of *Aurora-A* based on the ratio of 20q13.2 to 20q signals. Six out of seven esophageal cancer cell lines have amplified *Aurora-A*, three of which showed high amplification of *Aurora-A* (Table I).

We also found many isochromosomes of 20q13.2, which is a common feature of aneuploidy and gene amplification in cancer.

Amplification of Aurora-A in esophageal cancers. Quantitative real-time PCR was done to detect the amplification of *Aurora-A* in esophageal cancer tissues. Twenty-nine paired cancer samples and distant normal esophageal epithelium were investigated to evaluate the gene copy level of *Aurora-A* by using *GAPDH* as internal control.

GAPDH standard curve was constructed (Fig. 2) and then quantitative real-time PCR was done to detect the *GAPDH* gene copy number (Fig. 3). The same experiment was done to detect the *Aurora-A* gene copy number. The starting copy numbers were determined by the software Rotor-Gene 5.0,

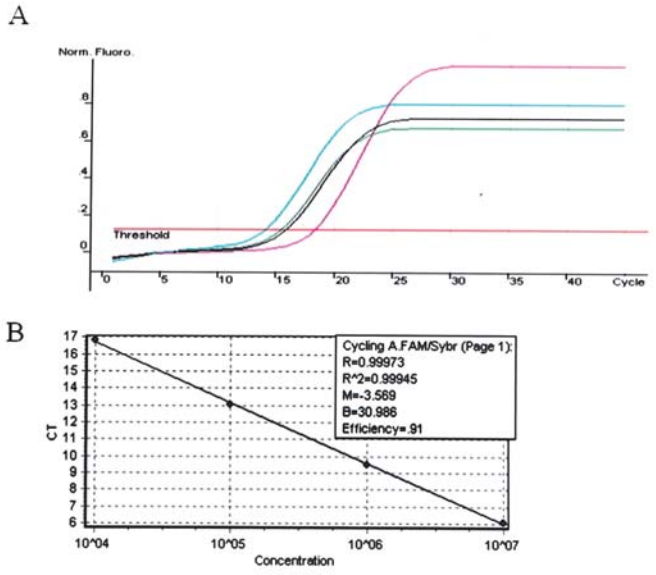

Figure 2. *GAPDH* standard curve by real-time PCR. Four real-time polymerase chain reactions were done with serial 10-fold dilutions of specific PCR products of *GAPDH* as template (A). The parameter C_t (threshold cycle) was determined by software Rotor-Gene 5.0, and the standard curve was constructed based on the C_t and the template concentration of the four samples (B).

and all the data were normalized to *GAPDH*. As showed in Table II, 27 of 29 (93.1%) cancer samples possessed higher *Aurora-A* gene copy level than their corresponding normal tissues. Therefore *Aurora-A* is amplified in ESCC.

Overexpression of Aurora-A in esophageal cancers and cancer cell lines. We tested the expression level of *Aurora-A* gene in 7 esophageal cancer cell lines by semi-quantitative reverse transcription PCR analysis and Western blot assay. We used HLF cell (human lung fibroblast cell) as a normal control. All esophageal cancer cell lines showed overexpression of *Aurora-A* compared with HLF cells at mRNA level, KYSE70, 410 and ESCC9706 also have higher expression of Aurora-A from protein level detection (Fig. 4).

We also detected mRNA expression level of *Aurora-A* in esophageal cancer samples (Fig. 5). Comparison of band densitometry between *Aurora-A* and ß-actin by software showed 71.1% (27/38) esophageal cancer samples had more than 1.5-fold increase of *Aurora-A* mRNA level in contrast to paired normal adjacent tissues (Table III).

In addition to RT-PCR analysis, ten cancer specimens were collected and assayed for Aurora-A expression by Western immunoblot analysis. Six Cancer samples showed increased

Table II. Amplification of *Aurora-A* in esophageal cancers.

	Amplification level (Am)	
	<1 (%)	>1 (%)
No. of patients	2 (6.9)	27 (93.1)

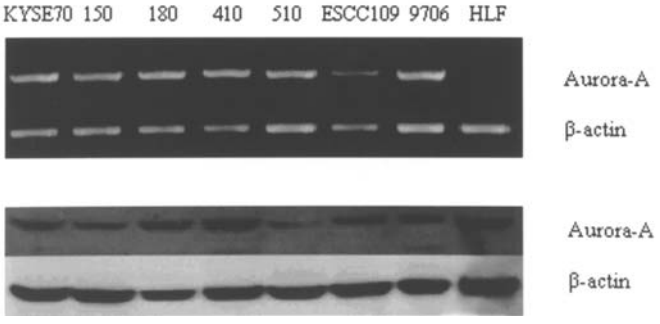

Figure 4. Overexpression of *Aurora-A* in esophageal cancer cell lines. Reverse transcription PCR showed increased *Aurora-A* mRNA level in 6 out of 7 esophageal squamous cell carcinomas (upper panel). HLF cell (human lung fibroblast cell) is the normal control. Protein level of Aurora-A was assayed by immunoblotting in 7 esophageal cancer cell lines by using HLF cells as normal control (lower panel). Six of seven esophageal cancer cell lines showed overexpression of *Aurora-A* at protein level. The membranes were reprobed with ß-actin as a loading control.

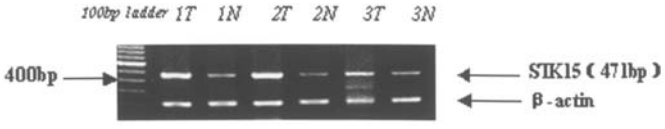

Figure 5. Expression of *Aurora-A* in esophageal cancers. Semi-quantitative RT-PCR of ESCC tissues. *Aurora-A* mRNA expression in esophageal cancers (T) was higher than the corresponding normal tissues (N). *Aurora-A* fragment (471 bp) was amplified with specific primers and ß-actin was used as internal control.

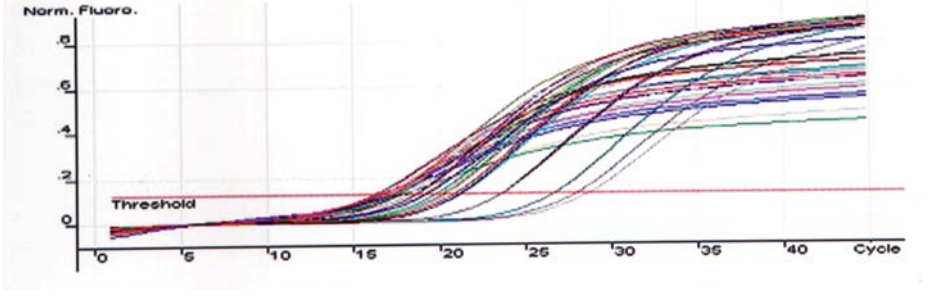

Figure 3. *GAPDH* gene copy number was detected by quantitative real-time PCR. Real-time PCR was performed to evaluate the *GAPDH* gene levels in esophageal cancers, the different color curves represent different samples.

Table III. Overexpression of *Aurora-A* in esophageal cancers.

	Elevated STK15 expression[a] (%)	No change of STK15 expression (%)	Total (%)
No. of patients	27 (71.1)	11 (28.9)	38 (100)

[a]The samples had >1.5-fold increase of *Aurora-A* mRNA level compared to that of the normal control.

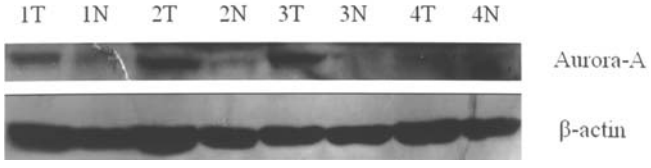

Figure 6. *Aurora-A* was overexpressed at the protein level in esophageal cancers. Analysis of Aurora-A protein expression in human ESCC tissues by Western blot assay. Cell lysates were prepared from clinical esophageal cancers and normal adjacent tissues (T, tumor; N, corresponding normal tissues). The same membrane was reprobed with anti-ß-actin as a loading control.

protein level of Aurora-A in human esophageal squamous cell carcinoma (Fig. 6).

Discussion

Esophageal cancer is the seventh leading cause of cancer death worldwide. In China, more than 90% of esophageal cancer is the squamous cell carcinoma type, and the area of the high incidence of ESCC is in the north. According to epidemiological studies, there are multiple etiological factors, such as nutritional deficiency, toxic stimuli, hot food ingestion and genetic background, contributing to the development of esophageal cancer. Because esophageal cancer usually is not diagnosed until the late stage and the disease has spread, prognosis of esophageal cancer is very poor. Fewer than 20% of people survive more than 5 years (22), therefore novel approaches to the early diagnosis and treatment of this malignant tumor are urgently needed, and understanding the molecular mechanism of human esophageal squamous cell carcinoma is the key to any such approach.

In our present study, we demonstrated amplification and overexpression of *Aurora-A*/*STK15* in esophageal cancer cell lines and esophageal cancer tissues.

Previous comparative genomic hybridization studies in esophageal cancer cell lines and tissues revealed that 20q amplification is common in esophageal cancers, but the genes involved in 20q gain are poorly identified. Given that the frequent 20q amplification in breast cancer and bladder cancer is often accompanied by the overexpression of *Aurora-A* (23,24), a mitosis regulating gene located on 20q13.2, we proposed *Aurora-A* as the possible target gene on amplified 20q in the esophageal cancer. In our present study, we identified frequent amplification of *Aurora-A* locus at 20q13.2 in seven esophageal cancer cell lines by dual-color FISH assay and in cancer samples by real-time PCR assay. Semi-quantitative RT-PCR was used to detect the expression of *Aurora-A* in esophageal cancer tissues. We found 27/38 (71.1%) esophageal cancers with elevated expression of *Aurora-A* when compared with corresponding normal tissues, which is consistent with other recent reports that *Aurora-A* is overexpressed in human ESCC (12,21). In addition, there are recent reports showing that the *Aurora-A* polymorphism is associated with ESCC risk (25,26). Although the relationship between the overexpression and polymorphism of *Aurora-A* needs to be further revealed, the polymorphism in *Aurora-A* gene was demonstrated to be associated with an increased risk in ESCC, as well as in breast cancer (25-27). Therefore, the studies, including ours, strongly suggested *Aurora-A* involvement in the Chinese esophageal cancer development and progression.

Aurora-A/*BTAK*/*STK15* gene, located in 20q13, frequently amplified chromosome region in many tumors including ESCC shown in our present study, belongs to a serine/threonine kinase family (Aurora kinase family) implicated in equal segregation of chromosomes between daughter cells. Aurora-A is important to centrosome duplication, maturation and accurate separation as well as to bipolar spindle assembly and stability. Its crucial role in the normal cell division suggested that change of *Aurora-A* expression might contribute to the aberrant cell growth in cancer. In fact, ectopic overexpression of *Aurora-A* in mammalian cells has been demonstrated to cause centrosome amplification, leading to aneuploidy. More importantly, overexpression of *Aurora-A* can lead to genetic instability and cause mammary tumor development in transgene mouse, which indicated that overexpressed *Aurora-A* could function as a potential oncogene causing carcinogenesis in many solid tumors.

Taken together, our present studies on the overexpression and amplification of Aurora-A in ESCC have provided indicative clues to revealing the mechanism of esophageal carcinogenesis and to find regimens to improve survival of esophageal cancer patients. Although the cause which triggers Aurora-A amplification and the mechanism involved during carcinogenesis in human solid tumors still need to be deeply investigated, the efficient therapy targeting Aurora-A in other malignant tumors (28-31) could also bring hope to esophageal cancer patients if the vital role that Aurora-A plays in esophageal cancer could be addressed further.

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