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Original Article

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Aurora-A modulates MMP-2 expression via AKT/NF-κB pathway in esophageal squamous cell carcinoma cells

Xiaoxia Wang[1,2,3,†,*], Xiaozhong Li[4,†], Chaohui Li[3], Chun He[3], Benhong Ren[3], Qing Deng[3], Wei Gao[1,2], and Binquan Wang[1,2,*]

[1]Department of Otolaryngology, Head & Neck Surgery, No. 1 Hospital, Shanxi Medical University, Taiyuan 030001, China, [2]Shanxi Key Laboratory of Otorhinolaryngology Head and Neck Cancer, Taiyuan 030001, China, [3]Department of Biochemistry and Molecular Biology, Shanxi Medical University, Taiyuan 030001, China, and [4]Department of Emergency, Shanxi Provincial People's Hospital, Taiyuan 030001, China

[†]These authors contributed equally to this work.
*Correspondence address. Tel: +86-351-4639235; Fax: +86-351-4639218; E-mail: wxiaoxia99007@126.com (X.W.)/ binquanwangsx@163.com (B.W.)

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Abstract

Esophageal squamous cell carcinoma (ESCC) is one of the most common malignancies. It is necessary to identify new markers for predicting tumor progression and therapeutic molecular targets. It has been reported that overexpressions of Aurora-A and matrix metalloproteinases 2 (MMP-2) may promote the malignant development of tumor. However, the relationship between Aurora-A and MMP-2 expression in tumor patients has not been investigated. In addition, the underlying mechanisms that Aurora-A regulates MMP-2 expression are still not fully elucidated. In this study, we demonstrated that Aurora-A and MMP-2 were overexpressed in ESCC tissues compared with paired normal adjacent tissues ($P < 0.0001$). Overexpression of Aurora-A was associated with the lymph node metastasis of ESCC ($P = 0.01$). Significantly, Aurora-A protein expression was positively correlated with MMP-2 protein expression in ESCC tissues ($r = 0.66$, $P < 0.0001$) as well as in ESCC cell lines. The level of Aurora-A expression was also positively correlated with the invasion capability of ESCC cells. Furthermore, Aurora-A overexpression significantly increased ESCC cell invasion by the upregulation of MMP-2 expression. In addition, Aurora-A overexpression promoted nuclear factor-kappaB (NF-κB) activation, and Aurora-A-mediated MMP-2 upregulation was abrogated by NF-κB inhibitor. Further analysis showed that activation of NF-κB was severely attenuated by AKT inhibitor in cells overexpressing Aurora-A. Taken together, these data indicate that Aurora-A overexpression upregulates MMP-2 expression through activating AKT/NF-κB signaling pathway in ESCC cells. These findings reveal that Aurora-A may be used as an important indicator for the judgment of malignant behavior of ESCC, and may be an attractive target for cancer therapy.

Key words: Aurora-A, matrix metalloproteinases 2, esophageal squamous cell carcinoma, nuclear factor-κB

Introduction

Esophageal squamous cell carcinoma (ESCC) is a common malignancy in the world and one of the main causes of cancer deaths [1]. The invasion and metastasis are the factors significantly affecting prognosis in ESCC patients. Therefore, a better understanding of the molecular mechanism of tumorigenicity and malignant development of ESCC may help to find new biomarkers for precise diagnosis and effective therapy.

Tumor invasion and metastasis are highly coordinated multistep and complex processes. The degradation of basement membranes and stromal extracellular matrix (ECM) by expressing and secreting various proteolytic enzymes plays a critical role in promoting tumor invasion and metastasis. The matrix metalloproteinases 2 (MMP-2, gelatinase A, 72 kDa), a member of the human zinc-dependent endopeptidase family, efficiently degrades components of the basement membrane and ECM such as Type IV collagen and fibronectin, and contributes to the invasion and metastasis of cancer cells [2–4]. Earlier reports have indicated that the increased expression level of MMP-2 is correlated with the invasive properties of various malignant tumors including ESCC [5–7], indicating that MMP-2 plays an important role in regulating malignant development of tumor cells. However, the precise regulatory mechanisms of MMP-2 in human cancer cells are not well defined.

Aurora-A (also known as STK15, BTAK, and Aurora 2), a member of mitotic serine/threonine Aurora kinase family, is responsible for the chromosome separation, maintenance of bipolar spindles and cytokinesis during mitosis. The overexpression of Aurora-A may lead to the amplification of centrosomes, aneuploidy, and subsequent tumor formation [8–10]. Furthermore, overexpression of Aurora-A has been observed in many human tumors, such as hepatocellular carcinoma [11], pancreatic cancer [12], bladder cancer [13], and breast cancer [14]. In ESCC, Aurora-A expression was found to be elevated at protein and mRNA levels in the tumor specimens, and the level of Aurora-A expression is strongly associated with invasion capability and decreased survival [15,16]. In addition, it has also been shown that the overexpression of Aurora-A may promote cell proliferation and prevent cell apoptosis in ESCC cells [17]. These findings indicate that Aurora-A might play an important role in the development of cell malignancy. Furthermore, some studies demonstrated that Aurora-A could regulate malignant behaviors of tumor cell by interacting with multiple critical molecules including nuclear factor-kappaB (NF-κB) [18]. NF-κB is an essential transcription factor that plays a pivotal role in tumor progression, and has been shown to regulate the expression of MMP-2 [19,20]. However, it is still not known if Aurora-A regulates MMP-2 through NF-κB in ESCCs.

The association between Aurora-A and MMP-2 expression has not been reported in ESCC patients. Therefore, the purpose of this study was to investigate the clinical significance of Aurora-A and MMP-2 expression, the association between Aurora-A and MMP-2 protein expression in ESCC patients, and the possible mechanism by which Aurora-A regulates MMP-2.

Materials and Methods

Immunohistochemical staining

Immunohistochemical staining was carried out on tissue section from the tissue microarray (TMA). TMA was constructed with a Beecher Instruments Tissue Array from 70 ESCC and paired normal adjacent tissue samples, which were obtained from the National Engineering Center for Biochip (Shanghai, China). Informed consent was obtained from all patients, and the protocol was approved by the Ethical Committee of National Engineering Center for Biochip. This study was performed in accordance with the ethical standards described in the Declaration of Helsinki. Sections were deparaffinized in xylene, rehydrated in graded alcohol, immersed in 10 mM sodium citrate buffer (pH 6.0), and pretreated in a microwave oven for 10 min. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide solution for 10 min. After being washed, the sections were treated with 1% bovine serum albumin for 30 min to block nonspecific reactions. Then, sections were incubated with antibody against Aurora-A (Cell Signaling, Beverly, USA) or MMP-2 (Santa Cruz Biotechnology, Santa Cruz, USA) at 4°C for overnight, followed by incubation with biotinylated secondary antibody and streptavidin–peroxidase complex. After visualization of the reaction with the diaminobenzidine (DAB) chromogen, the slides were counterstained with haematoxylin.

Evaluation of immunohistochemical staining

The TMA slides were independently evaluated by two experienced pathologists who had no prior knowledge of the clinical data using light microscopy. Consensus opinion of stain patterns was provided by these two pathologists. For Aurora-A, both cytoplasmic staining and nuclear staining were examined and evaluated. For MMP-2, only cytoplasmic staining was examined and evaluated. Immunohistochemical staining of cells was assessed according to stain intensity and extent (proportion of stained cells). The intensity of the immunostaining was graded using a 4-point scale: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The percentage of stained cells was assessed using a semiquantitative 4-point scale: 1, <25% positive tumor cells; 2, 25%–50% positive tumor cells; 3, 51%–75% positive tumor cells; and 4, >75% positive tumor cells. The results of staining intensity and extent were graded as follows: 0, negative (−); 1–4, weak positive (±); 5–8, moderate positive (+); and 9–12, strong positive (++). The scores of <5 were considered negative and the scores of ≥5 were considered positive for expression of Aurora-A or MMP-2 [15,21].

Preparation of cell lysates and nuclear fraction

Whole cell extracts were prepared by using protein lysis buffer (150 mM NaCl, 1% Triton X-100, and 50 mM Tris–HCl, pH 8.0) supplemented with 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin. The nuclear fraction was isolated using the nuclear extraction kit (KeyGEN Biotech, Nanjing, China) according to the manufacturer's instructions. Briefly, the cells were lysed in hypotonic buffer containing protease inhibitors for 15 min on ice. The suspension was vortexed and centrifuged at 14,000 *g* for 5 min at 4°C. The pellet (nuclear fraction) was lysed in nuclear extraction buffer in the presence of protease inhibitors. After centrifugation at 14,000 *g* for 10 min, the supernatant was stored at −80°C until use. Protein concentration was determined by the Bradford assay.

Cell culture and establishment of stably transfected cells

The KYSE150 and EC9706 ESCC cell lines (obtained from the Tumor Cell Bank of Chinese Academy of Medical Sciences, Beijing, China) were maintained with Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS). GFP-Aurora-A expression plasmid or control plasmid was

transfected into KYSE150 or EC9706 cell with Lipofectamine 2000 transfection reagent according to the manufacturer's instructions. Stable clones were selected with 0.4 mg/ml G418 (Invitrogen, Carlsbad, USA) for 2 weeks.

Western blot analysis

The cells were pretreated with or without pyrrollidine dithiocarbamate (PDTC, an inhibitor of NF-κB; Sigma, St Louis, USA) or LY294002 (AKT inhibitor; Calbiochem, San Diego, USA) for 48 h. Equivalent amounts of protein were electrophoresed on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose membranes (Millipore, Billerica, USA). After being blocked with 5% nonfat dry milk, the membranes were incubated overnight at 4°C with antibodies against Aurora-A, MMP-2, or NF-κB p65 (Abcam, Cambridge, UK), respectively. After being washed, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 2 h. Then, membranes were washed extensively and detected with electro-chemi-luminescence (ECL) reagent. Anti-β-actin antibody (Santa Cruz) or anti-proliferating cell nuclear antigen (anti-PCNA) antibody (Bioworld, Atlanta, USA) was used to detect β-actin and PCNA expressions which were used as loading controls. The protein bands were quantified using the Quantity One Software (Bio-Rad, Hercules, USA).

Cell invasion assay

Invasion assay was performed with Boyden chamber (Neuro Probe, Gaithersburg, USA) according to the manufacturer's instructions. The filters were coated with the Matrigel. The cells (2×10^4) in serum-free RPMI 1640 with or without MMP-2 inhibitor I (Calbiochem) were seeded into each upper chamber of the Transwell. RPMI 1640 containing 5% FBS was placed in the lower chamber and incubated at 37°C for 48 h. Cells that invaded to the lower surface of the membrane were stained with crystal violet and counted under a light microscope.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using Trizol Reagent (Invitrogen), and reverse-transcribed to cDNA using a commercial kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. For qPCR analysis, aliquot of double-stranded cDNA was amplified with primers using a SYBR Green polymerase chain reaction (PCR) kit (TaKaRa) in an ABI Prism 7500 sequence detector (Applied Biosystems, Foster City, USA). Primer sequences were as follows: *MMP-2* forward: 5′-GTGAAGTATGGGAACGCCGA-3′; reverse: 5′-AGAAGCCGT ACTTGCCATCC-3′, and *GAPDH* forward: 5′-GGCCTCCAAGG AGTAAGACC-3′; reverse: 5′-AGGGGTCTACATGGCAACTG-3′. *GAPDH* was served as an internal control. PCR conditions were as follows: 95°C for 2 min, 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s.

Immunofluorescent staining

The cells were grown in monolayer on glass slides at 37°C for 24 h and fixed with cold methanol for 30 min. Then, the cells were incubated with anti-NF-κB p65 subunit antibody overnight at 4°C. The cells were washed and incubated with tetramethylrhodamineisothiocyanate (TRITC)-conjugated secondary antibody for 1 h. After being washed, cells were stained with 4,6-diamidino-2-phenylindole (DAPI) and images were collected using a confocal fluorescence microscope (Olympus FV1000, Center Valley, USA).

Statistical analysis

SPSS statistical software package (Version 17.0) was used for all statistical calculations. The difference of Aurora-A or MMP-2 expression between ESCC and paired normal adjacent tissues as well as correlation between these protein expression and clinicopathologic variables were analyzed using Chi-square test or Fisher's exact test. The association between Aurora-A and MMP-2 expression was analyzed using Spearman's rank correlation. Student's *t*-test was used to analyze the statistical significance between groups. The results were expressed as the means ± SD. $P < 0.05$ was considered to be statistically significant.

Results

Expressions of Aurora-A and MMP-2 proteins in ESCC tissue

The expressions of Aurora-A and MMP-2 proteins were detected by immunohistochemical staining in 70 ESCC samples and paired normal adjacent tissues on TMA. As shown in **Fig. 1**, the staining of Aurora-A was negative in adjacent normal tissues (**Fig. 1A**), while ESCC tissues showed elevated expression of Aurora-A in both cytoplasm and nucleus of the ESCC cells, predominantly in cytoplasm (**Fig. 1B–D**). Thus, expression of Aurora-A was scored according to cytoplasmic and nuclear staining of the cancer cells, respectively. The immunohistochemical staining results were summarized in **Table 1**. In ESCC samples, 77% (54/70) cases exhibited positive Aurora-A cytoplasmic expression and 23% (16/70) cases showed negative cytoplasmic expression. In adjacent normal tissues, the only 21% (15/70) sample displayed Aurora-A positive cytoplasmic expression. There was a statistically significant difference between ESCC samples and paired normal adjacent tissues ($P < 0.0001$). However, Aurora-A nuclear staining did not show statistically significant difference between tumor tissues and adjacent normal tissues (data not shown). Additionally, negative cytoplasmic and

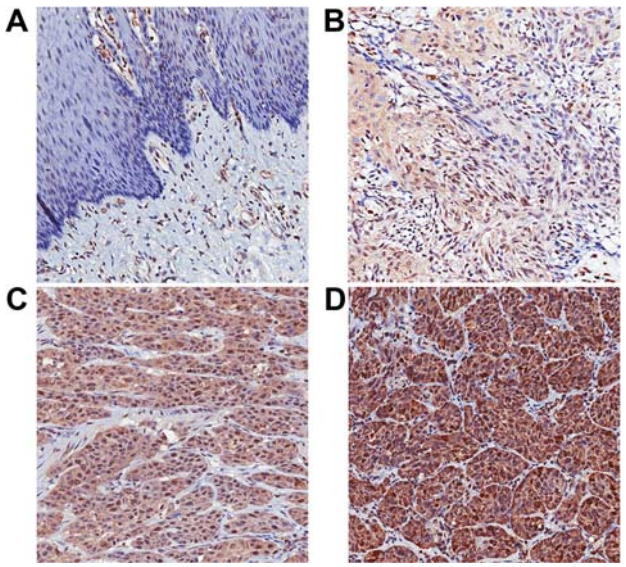

Figure 1. Representative immunohistochemical stainings of Aurora-A in ESCC and normal adjacent tissues on TMA (A) In normal adjacent esophageal tissues, Aurora-A showed negative staining. In ESCC tissues, Aurora-A staining was weakly (±) (B), moderately (+) (C), and strongly (++) (D) positive in both cytoplasm and nucleus of ESCC cells, predominantly in cytoplasm. Original magnification, ×200.

weakly nuclear staining of MMP-2 was observed in adjacent normal tissues (**Fig. 2A**). In contrast, MMP-2 cytoplasmic staining was upregulated in ESCC tissues (**Fig. 2B–D**). So MMP-2 expression was scored according to cytoplasmic staining. Of the 70 ESCC cases, MMP-2 expression was positive in 89% (62/70), and was higher than that in adjacent normal tissues (39%, 27/70) ($P < 0.0001$) (**Table 1**). These data indicate that Aurora-A and MMP-2 are significantly upregulated in ESCC and may play important roles in ESCC tumorigenesis and progression.

Overexpression of Aurora-A is associated with the lymph node metastasis of ESCC

Furthermore, the correlation between immunohistochemical staining of Aurora-A or MMP-2 expression and the clinical pathological features of ESCC was examined (**Table 2**). Our results showed that Aurora-A protein was more frequently expressed in tumors with lymph node metastasis (30 of 33, 91%) than in those without metastasis (24 of 37, 65%) ($P = 0.01$). However, no significant association was observed between Aurora-A expression and other clinicopathologic characteristics such as histologic grade ($P = 0.32$) and depth of invasion (T stage) ($P = 0.31$). Additionally, there was no statistically significant difference between MMP-2 expression and histologic grade ($P = 1.00$), T stage ($P = 0.24$), or lymph node metastasis ($P = 0.28$) of ESCC. These results suggest that Aurora-A plays an important role in tumor cell metastasis, and that Aurora-A might be an important biomarker in the developmental pathway of ESCC.

Table 1. The protein expressions of Aurora-A and MMP-2 in ESCC and normal adjacent tissues

	Normal tissue	Tumor tissue	*P* value[a]
	n = 70 (%)	*n* = 70 (%)	
Aurora-A			<0.0001
Negative	55 (79)	16 (23)	
Positive	15 (21)	54 (77)	
MMP-2			<0.0001
Negative	43 (61)	8 (11)	
Positive	27 (39)	62 (89)	

[a]Tumor tissue vs. normal adjacent tissue.

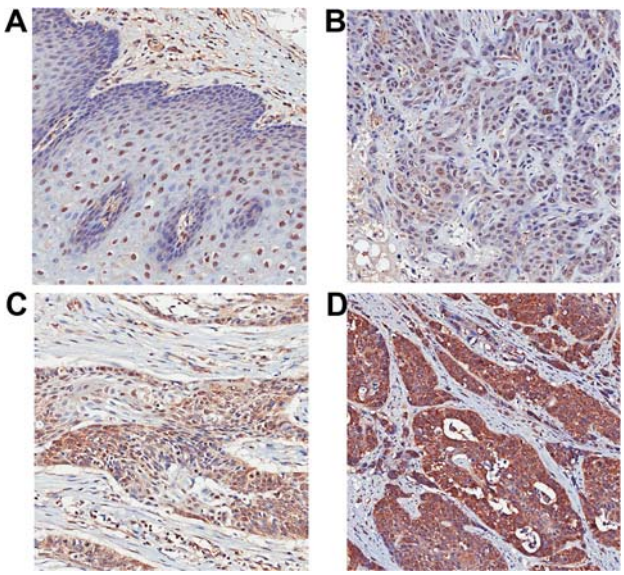

Figure 2. Representative immunohistochemical stainings of MMP-2 in ESCC and normal adjacent tissues on TMA (A) In normal adjacent esophageal tissues, MMP-2 showed negative cytoplasmic and weak nuclear staining. In ESCC tissues, MMP-2 staining was weakly (±) (B), moderately (+) (C), and strongly (++) (D) positive in the cytoplasm of ESCC cells. Original magnification, ×200.

Expression of Aurora-A is positively correlated with expression of MMP-2 in ESCC

As shown in **Table 3**, among the 70 ESCC tissues, 54 cases showed both Aurora-A and MMP-2 positive expressions in the cytoplasm, with a co-positive rate of 77% (54/70). Furthermore, Spearman's rank correlation analysis indicated that Aurora-A protein expression was strongly positively correlated with MMP-2 protein expression ($r = 0.66$, $P < 0.0001$). This result indicates that both Aurora-A and MMP-2 may play important roles in the malignant development of ESCC. An investigation into the combined expression of Aurora-A and MMP-2 may be useful in the diagnosis and therapy of ESCC.

Aurora-A expression is positively correlated with MMP-2 expression and cell invasive capability in ESCC cell lines

As the expression of Aurora-A protein was found to be correlated with MMP-2 expression in the patient cohort, we further analyzed their expressions in ESCC cell lines (**Fig. 3**). The results showed that Aurora-A and MMP-2 proteins were expressed ~3.5–5 folds higher in EC9706 cells than in KYSE150 cells (**Fig. 3A,B**). Significantly, EC9706 cell line with high expressions of Aurora-A and MMP-2 showed higher invasion potential (**Fig. 3C** and **Supplementary Fig. S1**). These data suggest that the level of Aurora-A expression is positively correlated with MMP-2 expression and invasion capability, which is consistent with our data from the patient cohort.

Aurora-A overexpression promotes ESCC cell invasion via upregulating MMP-2

In order to explore the molecular mechanism by which Aurora-A regulates MMP-2 expression in ESCC cells, Aurora-A was overexpressed in KYSE150 and EC9706 cells. Western blot analysis results showed that stable clones transfected with Aurora-A expression vector (named as Aur-1 and Aur-2) expressed fusion protein of GFP-Aurora-A, which was not seen in the control vector (named as Con) (**Fig. 4A**). Then, the effect of Aurora-A overexpression on MMP-2 was investigated. The results of western blot analysis (**Fig. 4A**) and qRT-PCR (**Fig. 4B**) showed that the protein and mRNA expression levels of MMP-2 in Aurora-A-overexpressing cells were higher than those in the control cells, which were consistent with previous studies that knockdown of Aurora-A expression could downregulate MMP-2 expression [22,23].

In addition, to verify that MMP-2 is required for Aurora-A-mediated cell invasion, Aurora-A-overexpressing cells were treated with MMP-2 inhibitor I. As shown in **Fig. 4C** and **Supplementary Figs. S2** and **S3**, the invasiveness of Aurora-A-overexpressing cells was markedly increased compared with vector control transfectants ($P < 0.01$), and the addition of MMP-2 inhibitor I dramatically suppressed the invasion of Aurora-A-overexpressing cells ($P < 0.01$).

Table 2. The relationship between Aurora-A or MMP-2 expression and patient clinicopathologic characteristics

	Aurora-A			MMP-2		
	Negative *n* (%)	Positive *n* (%)	*P* value	Negative *n* (%)	Positive *n* (%)	*P* value
Histologic grade						
Well and moderately	14 (26)	39 (74)	0.32	6 (11)	47 (89)	1.00
Poorly	2 (12)	15 (88)		2 (12)	15 (88)	
pT (depth of invasion)						
*T*1 + *T*2	4 (16)	21 (84)	0.31	1 (4)	24 (96)	0.24
*T*3 + *T*4	12 (27)	33 (73)		7 (16)	38 (84)	
pN (lymph node metastasis)						
N0	13 (35)	24 (65)	0.01	2 (5)	35 (95)	0.28
N1/N2/N3	3 (9)	30 (91)		6 (18)	27 (82)	

Table 3. The correlation of Aurora-A expression with MMP-2 expression in ESCC

	Aurora-A		Total	*r*	*P* value
	Negative	Positive			
MMP-2					
Negative	8	0	8	0.66	<0.0001
Positive	8	54	62		
Total	16	54	70		

These findings reveal that Aurora-A overexpression may increase ESCC cell invasion via upregulating MMP-2 expression.

Aurora-A overexpression upregulates MMP-2 expression via AKT/NF-κB pathway in ESCC cells

To identify whether the upregulating effect of Aurora-A overexpression on MMP-2 expression in ESCC cells is through activating NF-κB, the effect of Aurora-A overexpression on the activity of NF-κB was investigated. Western blot analysis demonstrated that the nuclear protein level of NF-κB p65 was significantly increased after Aurora-A overexpression (**Fig. 5A**), which was further confirmed by the results of immunofluorescent staining. As shown in **Fig. 5B**, the majority of NF-κB p65 was cytoplasmic staining in control cells and the p65 was shown to translocate into the nucleus in Aurora-A-overexpressing cells. These data indicate that Aurora-A overexpression significantly increases NF-κB activity. To determine whether the modulation of NF-κB activity by Aurora-A is responsible for regulation of MMP-2 production, PDTC, an inhibitor of NF-κB, was added to Aurora-A-overexpressing cells for 48 h. Western blot analysis revealed that MMP-2 expression was significantly decreased by PDTC in a dose-dependent manner compared with the untreated cells (**Fig. 5C**). These results suggest that Aurora-A overexpression induces MMP-2 expression in ESCC cells via activation of the NF-κB signaling pathway.

It has been shown that nuclear translocation of NF-κB is influenced by many signaling pathways, including AKT pathway [24]. Interestingly, our previous results indicated that Aurora-A overexpression increases MMP-2 expression through AKT signaling pathway [22]. Hence, to identify whether AKT pathway participates in Aurora-A-mediated NF-κB activation, AKT inhibitor was added to Aurora-A-overexpressing cells for 48 h. Western blot analysis demonstrated that inhibition of AKT significantly decreased nuclear accumulation of NF-κB in Aurora-A-overexpressing cells (**Fig. 5D**). This result suggests that Aurora-A may modulate the NF-κB activation by the AKT pathway. All these results suggest that Aurora-A overexpression may upregulate MMP-2 expression via activation of the AKT/NF-κB signaling pathway.

Discussion

Accumulating reports have demonstrated that Aurora-A expression is elevated in a wide variety of cancers and plays important roles in tumorigenicity and malignant development [11–16]. Our results showed that 77% (54 of 70 samples) of ESCC tumor tissues displayed positive expression of Aurora-A protein in the cytoplasm of tumor cells, which was in agreement with a previous study [15]. In addition, it has been reported that Aurora-A gene is localized in 20q13, a chromosome region amplified in a number of cancer cell lines and primary tumor types, and amplification of Aurora-A is detected in 93.1% ESCC samples [16]. These data indicate that gene amplification may be a major cause of Aurora-A overexpression in ESCC. However, some reports demonstrated that there is discordance between gene amplification and expression of Aurora-A in hepatocellular carcinoma [11], pancreatic cancer [12], and bladder cancer [13], indicating that other mechanisms are involved in the increased Aurora-A gene expression. So, the expression of Aurora-A is also likely to be regulated by other mechanisms such as transcriptional activation in ESCC. Furthermore, we found that overexpression of Aurora-A was associated with the lymph node metastasis of ESCC. Some reports also demonstrated that the upregulation of Aurora-A protein is correlated with lymph node metastasis in head and neck squamous cell carcinoma [25], non–small-cell lung cancer [26], and bladder cancer [27]. In addition, our previous study revealed that Aurora-A overexpression could promote ESCC cell migration and invasion and Aurora-A silencing could lead to the inhibition of cell invasion *in vitro* [22]. These data indicate that Aurora-A is the potential marker for ESCC tumor invasion and metastasis, and may play an important role in malignancy development of ESCC. Therefore, for the identification of new diagnostic markers and therapeutic targets, it is important to elucidate the molecular mechanism by which Aurora-A promotes cancer cell invasion and metastasis.

An increased expression of MMP-2 has frequently been detected in solid tumors, which can contribute to cancer invasion and metastasis through the degradation of ECM, which facilitates and accelerates the tumor cells to invade new tissues and enter the blood stream to travel to distant sites [2–7]. In this study, we found that MMP-2

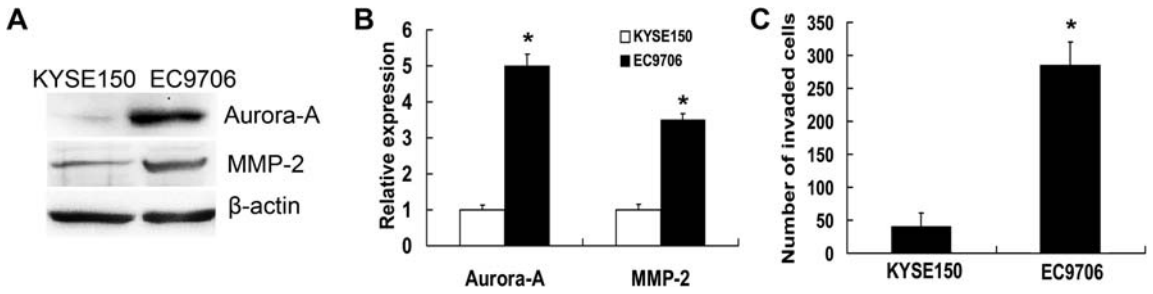

Figure 3. Expressions of Aurora-A and MMP-2 proteins as well as cell invasion capability in ESCC cell lines (A) The expression levels of Aurora-A and MMP-2 were detected by western blot analysis in KYSE150 and EC9706 cells. (B) The relative Aurora-A and MMP-2 protein levels were normalized to β-actin in KYSE150 and EC9706 cells. *$P < 0.05$, compared with KYSE150 cells. (C) The cell invasiveness was examined with Boyden chamber. *$P < 0.05$, compared with KYSE150 cells.

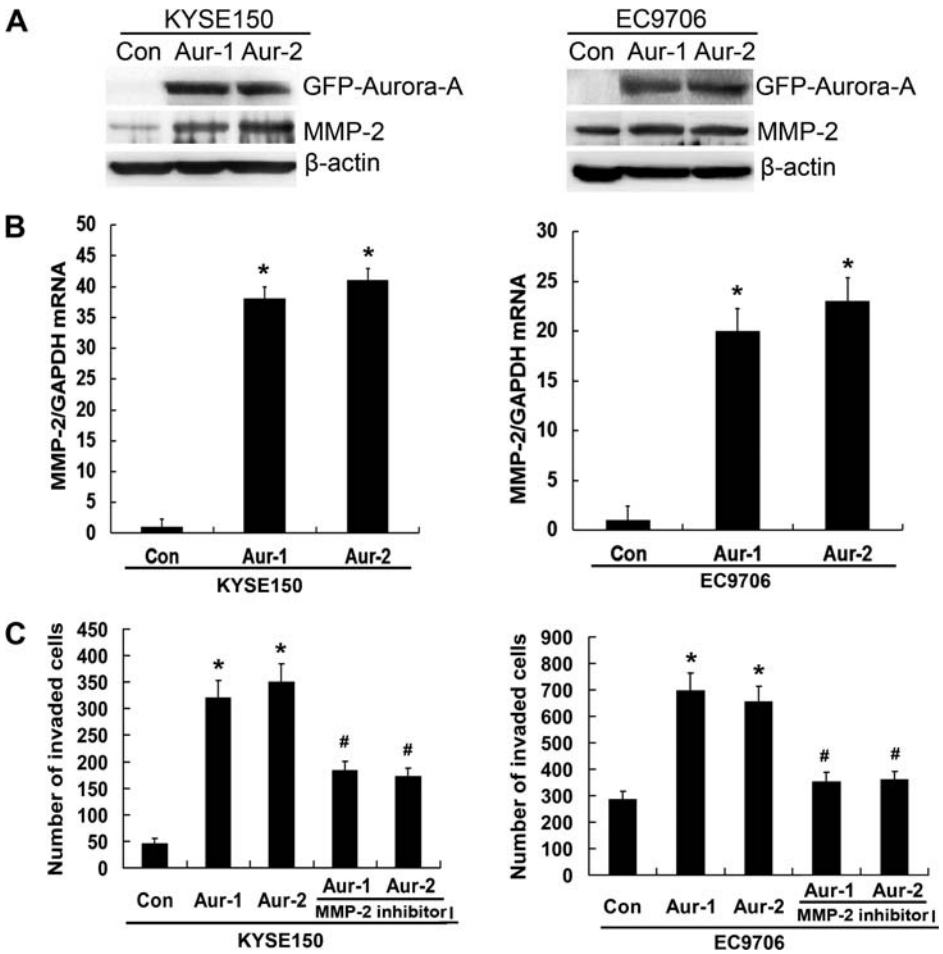

Figure 4. ESCC cell invasion is promoted by Aurora-A overexpression and attenuated by the MMP-2 inhibitor I (A) The levels of fusion protein GFP-Aurora-A and MMP-2 were detected by western blot analysis after stable transfection of GFP-Aurora-A expression vector in KYSE150 and EC9706 cells. (B) The mRNA level of MMP-2 was detected by qRT-PCR in Aurora-A-overexpressing and control cells. *$P < 0.05$, compared with the control cells. (C) The cell invasiveness was examined with Boyden chamber in the presence or the absence of 20 μM MMP-2 inhibitor I. *$P < 0.01$, compared with the control cells. $^{\#}P < 0.05$, compared with the Aurora-A-transfected cells.

was overexpressed in ESCC, which was consistent with previous studies [5,6]. However, no statistically significant difference was observed between MMP-2 expression and histologic grade, T stage, or lymph node metastasis of ESCC, which was not consistent with previous findings [6]. This discrepancy may be attributed to the difference in cancer patients recruited or to the relatively small number of clinical samples collected. Significantly, we found that co-expressions of Aurora-A and MMP-2 in the cytoplasm were positively correlated in ESCC tissues ($P < 0.0001$). Moreover, further analysis indicated that the expression level of Aurora-A was also positively correlated with MMP-2 expression and invasion capability in ESCC cells. Using specific MMP-2 inhibitor I, we confirmed that Aurora-A

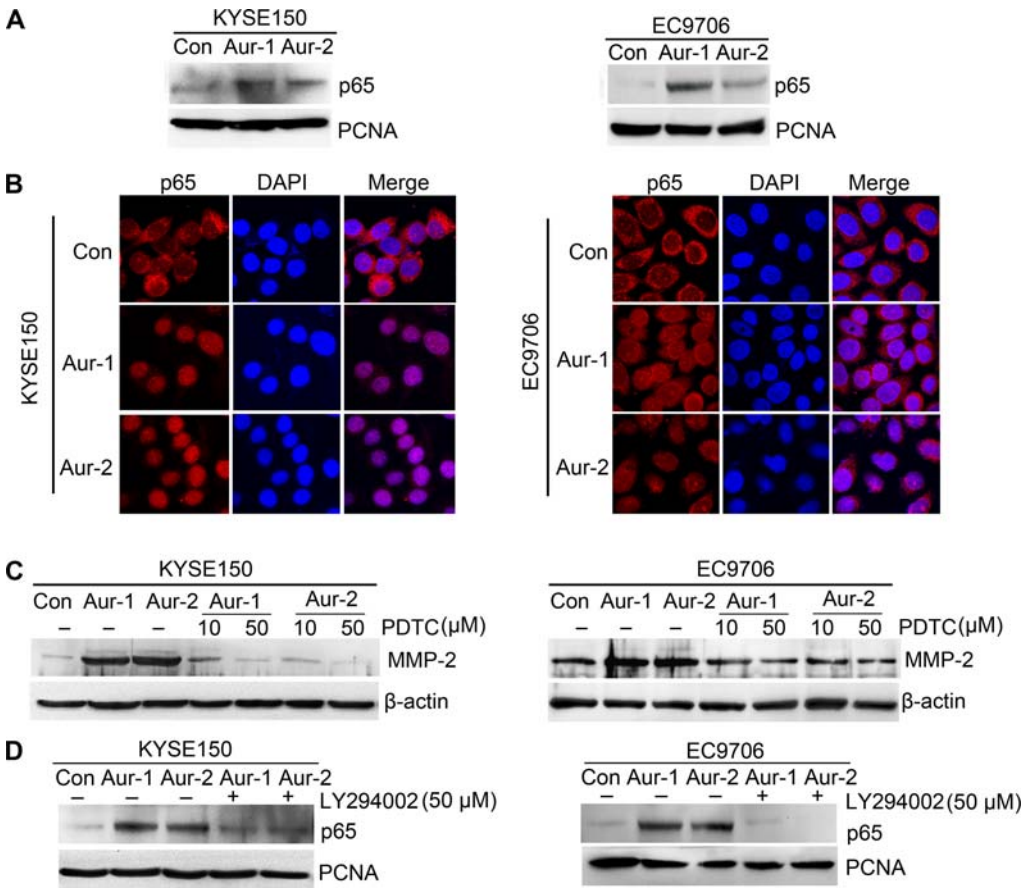

Figure 5. Aurora-A overexpression upregulates MMP-2 expression via AKT/NF-κB activation in ESCC cells (A) The nuclear protein level of NF-κB p65 was detected by western blot analysis in Aurora-A-overexpressing and control cells. (B) Cells grown on glass slides were fixed and incubated sequentially with anti-p65 and TRITC-conjugated (red staining) antibodies and cell nuclei were counterstained with DAPI (blue staining). Original magnification, ×200. (C) The protein level of MMP-2 was detected by western blot analysis after treatment with or without PDTC (10 and 50 μM) for 48 h in ESCC cells. (D) The nuclear protein level of NF-κB p65 was detected by western blot analysis after treatment with or without LY294002 (50 μM) for 48 h in ESCC cells.

overexpression promoted invasion of ESCC cells by upregulation MMP-2 expression. These results were consistent with a recent report showing that knockdown of Aurora-A decreases the migration and expression of MMP-2 in HEp-2 cells [23]. The data presented here indicate that Aurora-A and MMP-2 share a common molecular pathway in the progression of ESCC, and provide evidence for a critical role of MMP-2 in Aurora-A-induced invasion of ESCC cells.

NF-κB is a ubiquitous transcriptional factor which plays a central role in diverse cellular processes, including inflammation, immune response, and tumor progression [28–30]. NF-κB is present in the cytoplasm of unstimulated cells by interacting with IκBα. Following an appropriate stimulus, IκBα is phosphorylated and degraded, resulting in dissociation from NF-κB. Free NF-κB then translocates to the nucleus where it activates transcription of its target genes [28–30]. Several studies have reported that NF-κB pathway plays an important role in the regulation of MMP-2 expression. Li *et al.* [19] showed that IL-17A enhances hepatocellular carcinoma metastasis by the upregulation of MMP-2 expression via activating NF-κB signaling pathway. Bilandzic *et al.* [20] demonstrated that betaglycan suppresses MMP-2 expression through NF-κB pathway in human granulosa cell tumors. Additionally, Briassouli *et al.* [18] confirmed that Aurora-A is able to activate NF-κB via inducing IκBα phosphorylation. However, the potential role of NF-κB in Aurora-A-induced MMP-2 expression is still unknown. Our results demonstrated that overexpression of Aurora-A increased MMP-2 protein expression, and nuclear translocation of p65 protein which is an indicator of NF-κB activation. The incubation of Aurora-A-overexpressing cells with PDTC inhibited MMP-2 protein expression. Furthermore, we also found that LY294002 inhibited NF-κB activity by blocking nuclear translocation. In fact, several reports have shown that AKT plays an important role in tumor cell proliferation, migration, and invasion, and enhances the nuclear translocation of NF-κB [24]. We have also demonstrated that Aurora-A overexpression can increase MMP-2 expression through AKT signaling pathway [22]. Based on these data, we propose a molecular mechanism that Aurora-A may promote MMP-2 expression of ESCC cells at least partly through the activation of the AKT/NF-κB signaling pathway, which is important for the understanding of the malignant development of tumor cells.

In conclusion, our results indicate that overexpression of Aurora-A is associated with the lymph node metastasis of ESCC and the high expression of MMP-2 in ESCC tissues. In addition, Aurora-A overexpression significantly increases cell invasion by upregulating MMP-2 expression in ESCC cells. Furthermore, Aurora-A-mediated MMP-2 expression is dependent on AKT/NF-κB activation in ESCC cells. These findings reveal that Aurora-A may be a potential biomarker for the invasion and metastasis of ESCC, as well as an attractive target in the development of new therapies against cancer.

Supplementary Data

Supplementary data is available at *ABBS* online.

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