Carcinogenesis vol.30 no.5 pp.894–901, 2009
doi:10.1093/carcin/bgp064
Advance Access publication March 20, 2009

Overexpression of astrocyte elevated gene-1 (AEG-1) is associated with esophageal squamous cell carcinoma (ESCC) progression and pathogenesis

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Astrocyte elevated gene-1 (AEG-1), upregulated in various types of human cancers, has been reported to be associated with the carcinogenesis of human cancer. However, the functional significance of AEG-1 in human esophageal squamous cell carcinoma (ESCC) remains unknown. In the present study, we showed the expression of AEG-1 was markedly upregulated in esophageal cancer cell lines and surgical ESCC specimens at both transcriptional and translational levels. Immunohistochemical analysis revealed that 80 of 168 (47.6%) paraffin-embedded archival ESCC specimens exhibited high levels of AEG-1 expression. Statistical analysis suggested the upregulation of AEG-1 was significantly correlated with the clinical staging of the ESCC patients ($P = 0.001$), T classification ($P = 0.002$), N classification ($P = 0.034$), M classification ($P = 0.021$) and histological differentiation ($P = 0.035$) and those patients with high AEG-1 levels exhibited shorter survival time ($P < 0.001$). Multivariate analysis indicated that AEG-1 expression might be an independent prognostic indicator of the survival of patients with ESCC. Furthermore, we found that ectopic expression of AEG-1 in ESCC cells could significantly enhance cell proliferation and anchorage-independent growth ability. Conversely, silencing AEG-1 by short hairpin RNAi caused an inhibition of cell growth and anchorage-independent growth ability on soft agar. Moreover, we demonstrated that the upregulation of AEG-1 could reduce the expression of $p27^{Kip1}$ and induce the expression of cyclin D1 through the AKT/FOXO3a pathway. Our findings suggest that the AEG-1 protein is a valuable marker of ESCC progression and that the upregulation of AEG-1 plays an important role in the development and pathogenesis of human ESCC.

Introduction

Esophageal carcinoma, one of the most aggressive carcinomas of the gastrointestinal tract, is the sixth most common cause of cancer-related death in the world (1,2). Two main types of esophageal carcinoma, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma, with distinct etiological and pathological characteristics occur worldwide with variable geographic distribution. ESCC is more prevalent in China and other Asian countries (3,4). Despite rapid advancement of multimodal treatments for patients afflicted with ESCC, the prognosis has not been significantly improved, with an average 5 years survival rate of 10–20% (2,5,6). The prediction of ESCC clinical prognosis still depends on conventional pathologic variables such as tumor size, tumor grade, lymph node and distal metastasis status (7–9). Therefore, it is of great clinical value to find sensitive and specific early biomarkers for the diagnosis and prognosis of this malignancy, as well as novel therapeutic strategies.

Novel oncoprotein astrocyte elevated gene-1 (AEG-1), originally identified as a protein induced in primary human fetal astrocytes infected with human immunodeficiency virus (HIV)-1 or treated with HIV gp120 or tumor necrosis factor-α, has been demonstrated to be associated with the initiation and progression of cancer, the abrogation of cancer cell apoptosis and the induction of metastasis (10–12). It has been reported that ectopic expression of AEG-1 could augment anchorage-independent growth of non-tumorigenic melanocytes and astrocytes (10,12). Upregulation of AEG-1 could inhibit apoptosis induced by serum starvation in primary human fetal astrocyte immortalized astrocytes, whereas AEG-1 knockdown reduces cell viability and promotes apoptosis in prostate cancer cells (13,14). The oncogenic feature of AEG-1 has also been linked to tumor development and progression. It has been shown that the upregulation of AEG-1 increases and that the downregulation of AEG-1 decreases breast cancer cell metastasis in the lung (15). In addition, the overexpression of AEG-1 increases the migratory and invasive properties of glioma cells, whereas the depletion of endogenous AEG-1 in glioma cells significantly inhibits their migration and invasion (11,16). All these studies implicate AEG-1 in the oncogenesis of several malignancies. Indeed, the expression level of AEG-1 has been frequently found to be upregulated in various tumor types, including breast cancer, multiform glioblastoma, melanoma and prostate cancer (10,12,14,15). AEG-1 expression has been suggested to be an independent prognostic factor of breast cancer patient outcomes. Patients with higher AEG-1 expression had shorter overall survival time, whereas patients with lower AEG-1 expression had better survival (17).

In the present study, we found that the expression of AEG-1 was upregulated in ESCC cells and surgical specimens of ESCC. Importantly, the overexpression of AEG-1 is correlated with the clinical staging, tumor–node–metastasis classification and histological classification of the disease. Multivariate analysis revealed that AEG-1 might be an independent biomarker for the prediction of ESCC prognosis. Moreover, we demonstrated that a deregulation of AEG-1 expression was associated with the proliferation and tumorigenicity of ESCC cells, which may, in turn, be associated with the upregulation of cyclin D1 and the downregulation of $p27^{Kip1}$ though the AKT/FOXO3a pathway. Taken together, our results suggest that AEG-1 plays a significant role in human ESCC progression and pathogenesis.

Abbreviations: AEG-1, astrocyte- elevated gene-1; ESCC, esophageal squamous cell carcinoma; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MOD, mean optical density; NEEC, normal esophageal epithelial cell; PCR, polymerase chain reaction; SI, staining index.

[†]These authors contributed equally to this work.

Materials and methods

Cell lines

Primary cultures of normal esophageal epithelial cells (NEECs) were established from fresh biopsies of the adjacent non-cancerous esophageal tissue, which is over 5 cm from the cancerous tissue, according to the previous report (18). In general, esophageal biopsy was split into two pieces; first piece would be used for establishing esophageal epithelial cells. Second piece would be examined whether it is non-cancerous esophageal tissue by routine histopathological analysis, which used to confirm that the esophageal epithelial cells established from above-mentioned first piece are NEEC. The NEECs were grown at 37°C and 5% CO2 with keratinocyte serum free medium, with 40 μg/ml bovine pituitary extract, 1.0 ng/ml epidermal growth factor, 100 U/ml penicillin and 100 μg/ml streptomycin. The esophageal cancer cell lines, including Eca-109, TE-1 and Kyse140 (Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai, China), were

grown in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 μg/μl streptomycin and 100 μg/μl penicillin in a humidified incubator containing 5% CO_2 at 37°C.

Patient information and tissue specimens

This study was conducted on a total of 168 paraffin-embedded ESCC samples, which were histopathologically and clinically diagnosed at the Sun Yat-sen University Cancer Center from 2001 to 2003. For the use of these clinical materials for research purposes, prior patient consent and approval from the Institutional Research Ethics Committee were obtained. Clinical information on the samples is summarized in supplementary Table 1 (available at *Carcinogenesis* Online). Clinicalpathological tumor-node-metastasis staging was determined by the extent of tumor invasion in the esophageal wall and lymphatic and venous invasion status according to the criteria proposed by International Union Against Cancer criteria (19). Four biopsies of NSCC tissues and the matched adjacent non-cancerous esophageal tissues were frozen and stored in liquid nitrogen until further use.

RNA extraction, reverse transcription and real-time PCR

Total RNA from cultured cell and surgically obtained tumor tissues was extracted using the Trizol reagent according to the manufacturer's instruction. Real-time reverse transcription–polymerase chain reaction (PCR) primers and probes were designed with the assistance of the Primer Express v 2.0 software (Applied BioSystems, Foster, CA). Sequences of the primers are: *AEG-1*, 5′-AAATAGCCAGCCTATCAAGACTC-3′ (forward); *AEG-1*, 5′-TTCAGACTTGGTCTGTGAAGGAG-3′ (reverse); *AEG-1* probe, 5′-(FAM) CCACCTGCTACTTCTACCGAGCCAT (TAMRA)-3′; glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), 5′-GACTCATGACCACAGTCCATGC-3′ (forward); *GAPDH*, 5′-AGAGGCAGGGATGATGTTCTG-3′ (reverse) and *GAPDH* probe, 5′-(FAM) CATCACTGCCACCCAGAAGACTGTG (TAMRA)-3′. Expression data were normalized to the geometric mean of housekeeping gene *GAPDH* to control the variability in expression levels and analyzed using the $2^{-\Delta\Delta CT}$ method described by the previous report (20).

Vectors and retroviral infection

pMSCV/AEG-1-overexpressing human AEG-1 was generated by subcloning the PCR-amplified human AEG-1 coding sequence into pMSCV vector. To silence endogenous AEG-1, two RNAi oligonucleotides were cloned into the pSuper-retro-puro vector to generate pSuper-retro-AEG-1-RNAis, respectively (17). Retroviral production and infection were performed as described previously (17). Stable cell lines-expressing AEG-1 or AEG-1 RNAis were selected for 10 days with 0.5 μg/ml puromycin 48 h after infection. After 10 days selection, the Eca-109 cell lysates prepared from the pooled population of cells in sample buffer were fractionated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis for the detection of AEG-1 protein level.

Western blotting

Western blotting was performed according to standard methods as described previously (17), using anti-AEG-1 (Invitrogen), anti-AKT, anti-phospho-AKT (Ser^{473}), anti-GSK3β, anti-phospho-GSK-3β (Ser^{9}), anti-FoxO3a, anti-phospho-FoxO3a (Ser^{253}), anti-Cyclin D1 and anti-p27 antibodies (Cell Signaling, Danvers, MA).

Immunohistochemistry

Immunohistochemical analysis was performed to study altered protein expression in 168 paraffin-embedded ESCC tissues. The procedures were carried out similarly to previously described methods (17). In brief, paraffin-embedded specimens were cut into 4 μm sections and baked at 65°C for 30 min. The sections were deparaffinized with xylenes and rehydrated. Sections were submerged into ethylenediaminetetraacetic acid antigenic retrieval buffer and microwaved for antigenic retrieval. The sections were treated with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity, followed by incubation with 1% bovine serum albumin to block the non-specific binding. Rabbit anti-AEG-1 (1:500; Zymed, San Francisco, CA) was incubated with the sections overnight at 4°C. For negative controls, the rabbit anti-AEG-1 antibody was replaced with normal goat serum or the rabbit anti-AEG-1 antibody was blocked with a recombinant AEG-1 polypeptide by coincubation at 4°C overnight preceding the immunohistochemical staining procedure. After washing, the tissue sections were treated with biotinylated anti-rabbit secondary antibody (Zymed), followed by further incubation with streptavidin–horseradish peroxidase complex (Zymed). Tissue sections were then immersed in 3.3′-diaminobenzidine and counterstained with 10% Mayer's hematoxylin, dehydrated and mounted.

The degree of immunostaining of formalin-fixed, paraffin-embedded sections was viewed and scored separately by two independent investigators, who were blinded to the histopathological features and patient data of the samples, and the scores were determined by combining the proportion of positively stained tumor cells and the intensity of staining. Scores given by the two independent investigators were averaged for further comparative evaluation of the AEG-1 expression. Tumor cell proportion was scored as follows: 0 (no positive tumor cells); 1 (<10% positive tumor cells); 2 (10–35% positive tumor cells); 3 (35–70% positive tumor cells) and 4 (>70% positive tumor cells). Staining intensity was graded according to the following criteria: 0 (no staining); 1 (weak staining = light yellow); 2 (moderate staining = yellow brown) and 3 (strong staining = brown). Staining index (SI) was calculated as the product of staining intensity score and the proportion of positive tumor cells. Using this method of assessment, we evaluated AEG-1 expression in benign esophageal epithelia and malignant lesions by determining the SI, with scores of 0, 1, 2, 3, 4, 6, 9 or 12. The cutoff value for high and low expression level was chosen on the basis of a measure of heterogeneity with the log-rank test statistical analysis with respect to overall survival. An optimal cutoff value was identified: an SI score of ≥6 was used to define tumors with high AEG-1 expression, and an SI score of ≤4 was used to indicate low AEG-1 expression.

Immunohistochemical (IHC) staining for protein expression in tumor and normal tissues was quantitative analyzed with the AxioVision Rel.4.6 computerized image analysis system assisted with the automatic measurement program (Carl Zeiss, Oberkochen, Germany). Briefly, the stained sections were evaluated at ×200 magnification, and 10 representative staining fields of each section were analyzed to verify the mean optical density (MOD), which represents the strength of staining signals as measured per positive pixels. The MOD data were statistically analyzed using *t*-test to compare the average MOD difference between different groups of tissues, and $P < 0.05$ was considered significant.

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay

Cells were seeded on 96-well plates at initial density of (0.2×10^4 per well). At each time point, cells were stained with 100 μl sterile 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide dye (0.5 mg/ml, Sigma, St Louis, MO) for 4 h at 37°C, followed by removal of the culture medium and addition of 150 μl of dimethyl sulphoxide (Sigma). The absorbance was measured at 570 nm, with 655 nm as the reference wavelength. All experiments were performed in triplicates.

Anchorage-independent growth ability assay

Five hundred cells were trypsinized and suspended in 2 ml complete medium plus 0.3% agar (Sigma). The agar–cell mixture was plated on top of a bottom layer with 1% complete medium agar mixture. After 10 days, viable colonies that contained >50 cells or were larger than 0.5 mm were counted. The experiment was performed for three independently times for each cell line.

Colony formation assays

Cells were plated on 60 mm plates (0.5×10^3 cells per plate) and cultured for 10 days. The colonies were stained with 1% crystal violet for 30 s after fixation with 10% formaldehyde for 5 min.

Statistical analysis

All statistical analyses were carried out using the SPSS 13.0 statistical software package. The chi-square test and Fisher's exact test were used to analyze the relationship between AEG-1 expression and clinicopathological characteristics. Bivariate correlations between study variables were calculated by Spearmans rank correlation coefficients. Survival curves were plotted by the Kaplan–Meier method and compared using the log-rank test. Survival data were evaluated using univariate and multivariate Cox regression analyses. In all cases, $P < 0.05$ was considered statistically significant.

Results

Upregulation of AEG-1 in ESCC

Western blot analysis revealed that AEG-1 protein was highly expressed in all esophageal cancer cell lines, including Eca-109, Kyse-140 and TE-1, whereas it was weakly detected in NEECs (Figure 1A). In parallel with the upregulation of AEG-1 protein, real-time PCR results showed that all ESCC lines exhibited significantly higher (up to 14-fold) levels of AEG-1 messenger RNA compared with the NEEC cells.

To verify whether the upregulation of AEG-1 is clinically correlated with ESCC progression, comparative analysis of AEG-1 expression was conducted on four cases of paired primary ESCC tissue and adjacent non-cancerous tissue. Real-time PCR analysis revealed that the tumor:normal ratio of AEG-1 message expression could be as high as 22.5-fold in one case of four paired primary ESCC tissues (Figure 1C). The expression of AEG-1 protein was also found to be upregulated in all four human primary ESCC tissues compared with

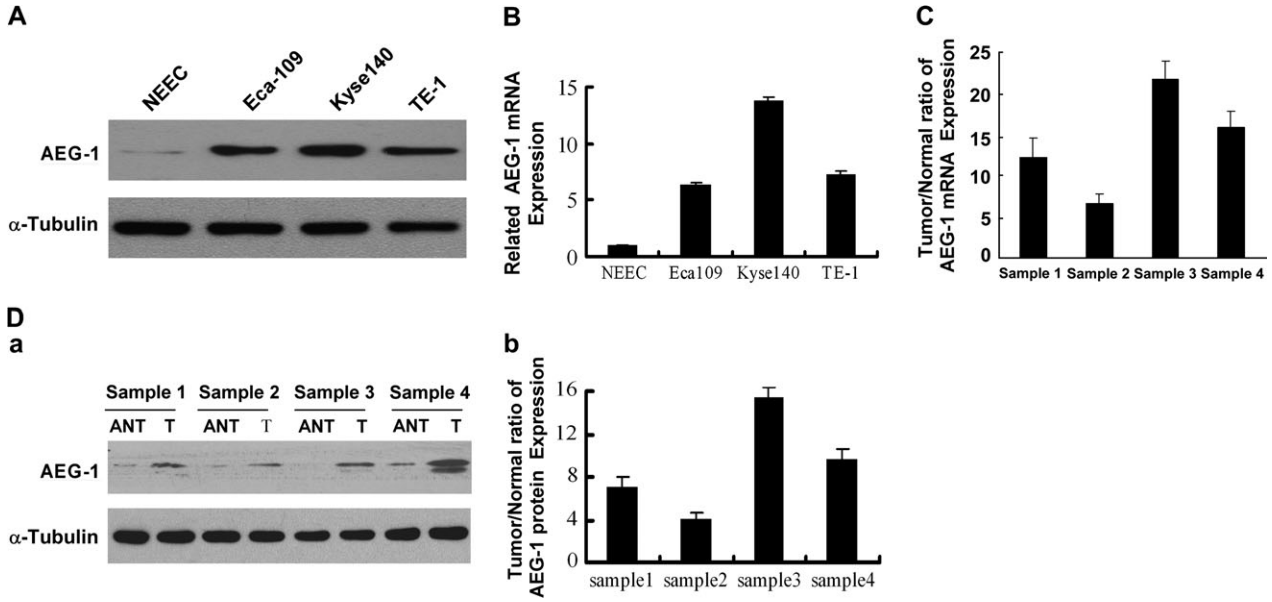

Fig. 1. Expression of AEG-1 is elevated in ESCC. (**A**) Expression of AEG-1 protein in normal human esophageal epithelial cells (NEEC) and cultured ESCC cell lines Eca-109, Kyse-140 and TE-1. Expression levels were normalized with α-tubulin. (**B**) Quantification of AEG-1 mRNA in NEEC and cultured ESCC cell lines. Expression levels were normalized for *GAPDH*. Error bars represent standard deviations calculated from three parallel experiments. (**C**) Real-time PCR analysis of AEG-1 expression in each of the primary ESCC (T) and esophageal adjacent non-cancerous tissues (ANT) paired from the same patient. *GADPH* was used as a loading control. (**D**) Expression (a) and quantification (b) of AEG-1 protein in each of the primary ESCC (T) and esophageal ANT paired from the same patient by western blotting. Expression levels were normalized with α-tubulin.

their matched adjacent non-cancerous tissues, using western blotting (Figure 1Da). Importantly, protein quantification showed that all four tumors displayed a >4-fold increase of AEG-1 protein compared with tissues adjacent to the tumors (Figure 1Db). It is of note that the level of AEG-1 protein in the esophageal cancer cells and clinical ESCC tissues was correlated with the messenger RNA expression level, indicating that the upregulation of AEG-1 in ESCC may be primarily caused by transcriptional upregulation.

Overexpression of AEG-1 in archived ESCC tissues

To further examine whether AEG-1 protein upregulation is associated with clinicopathological characteristics of ESCC, 168 paraffin-embedded, archived ESCC tissues were examined by IHC staining with an antibody against human AEG-1, including nine cases of stage I, 73 cases of stage IIa, 14 cases of stage IIb, 62 cases of stage III and 10 cases of stage IV tumors. As summarized in Table I, AEG-1 protein was detected in 156 of 168 (92.9%) cases. As shown in Figure 2A, AEG-1 was found to be upregulated in ESCC compared with normal esophageal tissues. High levels of AEG-1 expression were present in areas containing tumor cells of the primary ESCCs. In contrast, AEG-1 was barely detectable or only marginally detectable in the adjacent non-cancerous tissues in all tumor sections. Quantitative analysis indicated that the average MODs of AEG-1 staining in clinical stage I–IV primary tumors were statistically significantly higher than those in adjacent non-cancerous esophageal tissues ($P < 0.001$, Figure 2B). Taken together, these observations suggested that high levels of AEG-1 expression were associated with the clinical development of primary ESCC.

Relationship of AEG-1 upregulation with the clinical features of ESCC

Statistical analyses were done to examine the correlation between the expression of AEG-1 protein and the clinicopathological characteristics of ESCC. As shown in Table I, AEG-1 expression strongly correlated with gender ($P = 0.041$), clinical staging ($P = 0.001$), T classification ($P = 0.002$), N classification ($P = 0.034$), M classification ($P = 0.021$) and histological differentiation ($P = 0.035$) of patients with ESCC, whereas it was not associated with age ($P = 0.443$). Spearman correlation analysis was further performed to confirm the correlation between AEG-1 expression and clinicopathological features. As shown in supplementary Table 2 (available at *Carcinogenesis* Online), Spearman correlations of AEG-1 expression levels to gender, clinical staging, T classification, N classification, M classification and histological differentiation were 0.157 ($P = 0.042$), 0.288 ($P < 0.001$), 0.239 ($P = 0.002$), 0.164 ($P = 0.034$), 0.179 ($P = 0.021$) and 0.179 ($P = 0.020$), respectively. Taken as a whole, these observations supported the notion that the increased AEG-1 expression is associated with ESCC clinical progression.

Patient survival analysis indicated a clear inverse correlation between AEG-1 protein expression level and the overall survival time of ESCC patients ($P = 0.001$), with a correlation coefficient of −0.247 (supplementary Table 3 is available at *Carcinogenesis* Online). Moreover, the log-rank test showed that survival time was significantly different between the low and high AEG-1 expression groups ($P = 0.004$). As shown in Figure 2C, the cumulative 5 years survival rate was 40.7% in the low AEG-1 protein expression group (95% confidence interval, 0.5095–0.3044), whereas it was only 22.6% in the high expression group (95% confidence interval, 0.3177–0.1343).

Deregulation of AEG-1 enhance proliferation and tumor-promoting activity of ESCC cells

To further investigate the biological role of AEG-1 expression in ESCC progression, ESCC cell line Eca-109 was established to stably overexpress AEG-1 (Figure 3A). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay results indicated that AEG-1-infected Eca-109 cells grew faster, with ∼2.2-fold more cells than the control by day 5 after plating (Figure 3B). This phenomenon was further confirmed by a colony formation assay (Figure 3C). In consistent with above-mentioned results, the biological function of AEG-1 on proliferation was further confirmed in the NEEC (supplementary Figure 1A and B is available at *Carcinogenesis* Online). Previously, we showed that expression of AEG-1 is positively correlated with tumor classification ($P = 0.004$), and the effect of AEG-1 on the tumor-promoting activity of ESCC cells was further examined by

Table I. Correlation between AEG-1 expression and the clinicopathological characteristics of the ESCC patients

Characteristics	AEG-1		Chi-square test *P*-value	Fisher's exact test *P*-value
	Low expression no. (%)	High expression no. (%)		
Gender				
Male	62 (74.5)	67 (80.3)	0.041	0.046
Female	26 (25.5)	13 (19.7)		
Age (years)				
≥60	40 (40.2)	25 (27.3)	0.059	0.081
<60	48 (59.8)	55 (72.7)		
Clinical stage				
I	9 (7.8)	0 (1.5)	0.001	<0.001
IIa	43 (48.9)	30 (37.5)		
IIb	8 (9.1)	6 (7.5)		
III	27 (34.2)	35 (40.9)		
IV	1 (1.0)	9 (13.6)		
T classification				
T_1	10 (9.8)	1 (1.5)	0.002	0.001
T_2	27 (30.4)	19 (22.7)		
T_3	51 (57.8)	54 (69.7)		
T_4	0 (2.0)	6 (6.1)		
N classification				
N_0	54 (59.8)	36 (43.9)	0.034	0.044
N_1	34 (40.2)	44 (56.1)		
M classification				
Yes	1 (1.0)	7 (10.6)	0.021	0.028
No	87 (99.0)	73 (89.4)		
Expression of Ki67				
Low	47 (53.4)	30 (37.5)	0.039	0.045
High	41 (46.6)	50 (62.5)		
Histological differentiation				
Well	40 (45.5)	21 (26.3)	0.035	0.037
Moderate	28 (31.8)	34 (42.5)		
Poor	20 (22.7)	25 (31.3)		

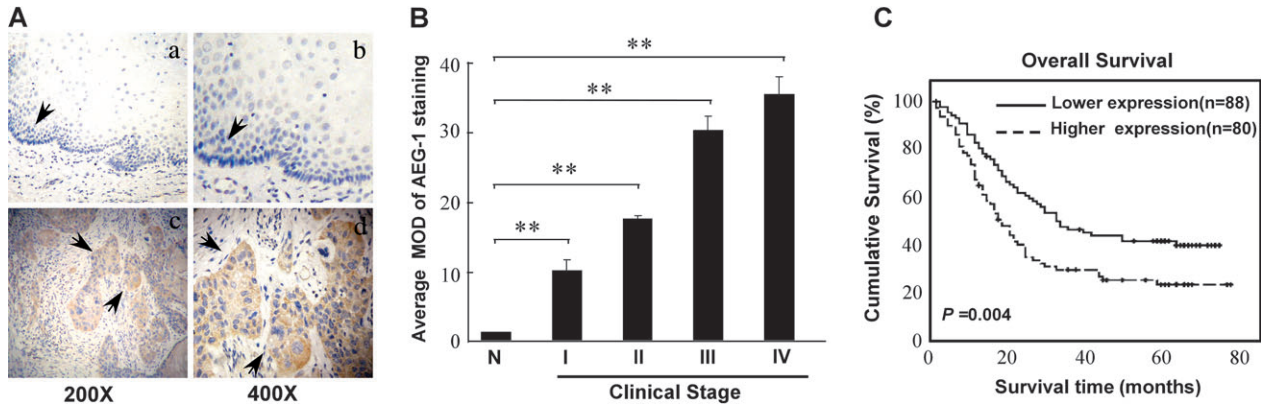

Fig. 2. AEG-1 protein is overexpressed in ESCC histopathological sections and AEG-1 expression inversely correlates with survival of patients afflicted with ESCC. (**A**) Representative images from IHC assays of 168 archived ESCC cases, among which 80 cases were positive for AEG-1. (a) and (b) AEG-1 expression in normal esophageal epithelial cells was only marginally detectable (Arrows: a, ×200; b, ×400). (c) and (d) AEG-1 expression in the primary lesions of ESCC (Arrows: c, ×200; d, ×400). (**B**) The average MOD of AEG-1 staining between the adjacent esophageal tissues (four cases) and different clinical stage ESCC (randomly picked 10 cases per stage) were statistically quantified. The average MOD of AEG-1 staining increases as ESCC progresses to more advanced stages ($P < 0.001$). Error bars represent mean ± SD from three independent experiments. (**C**) Survival curves of patients inflicted with ESCC with low expression versus high expression of AEG-1 ($P = 0.004$, log-rank test). The cumulative 5 years survival rate was 40.7% (95% confidence interval, 0.5095–0.3044) in the low AEG-1 protein expression group ($n = 88$) (bold line), but it was only 22.6% (95% confidence interval, 0.3177–0.1343) in the high expression group ($n = 80$) (dotted line). *P*-values were calculated by log-rank test.

an anchorage-independent growth ability assay. As shown in Figure 3D, the upregulation of AEG-1 could significantly increase the anchorage-independent growth ability of Eca-109 cells. Around six times more colonies were formed by Eca-109/AEG-1 cells than that in Eca-109/vector cells.

Furthermore, the impact of AEG-1 expression on ESCC proliferation was evaluated in AEG-1 knockdown cells (Figure 4A). As shown in Figure 4B, the depletion of AEG-1 expression caused significantly compromised viability in Eca-109 cells. The anchorage-independent growth ability assay revealed that silencing endogenous AEG-1 in Eca-109 cells could lead to decreases of both colony number and colony size (Figure 4C). Taken together, these results suggested that AEG-1 is essential for the proliferation in ESCC cells and may be involved in ESCC tumor formation.

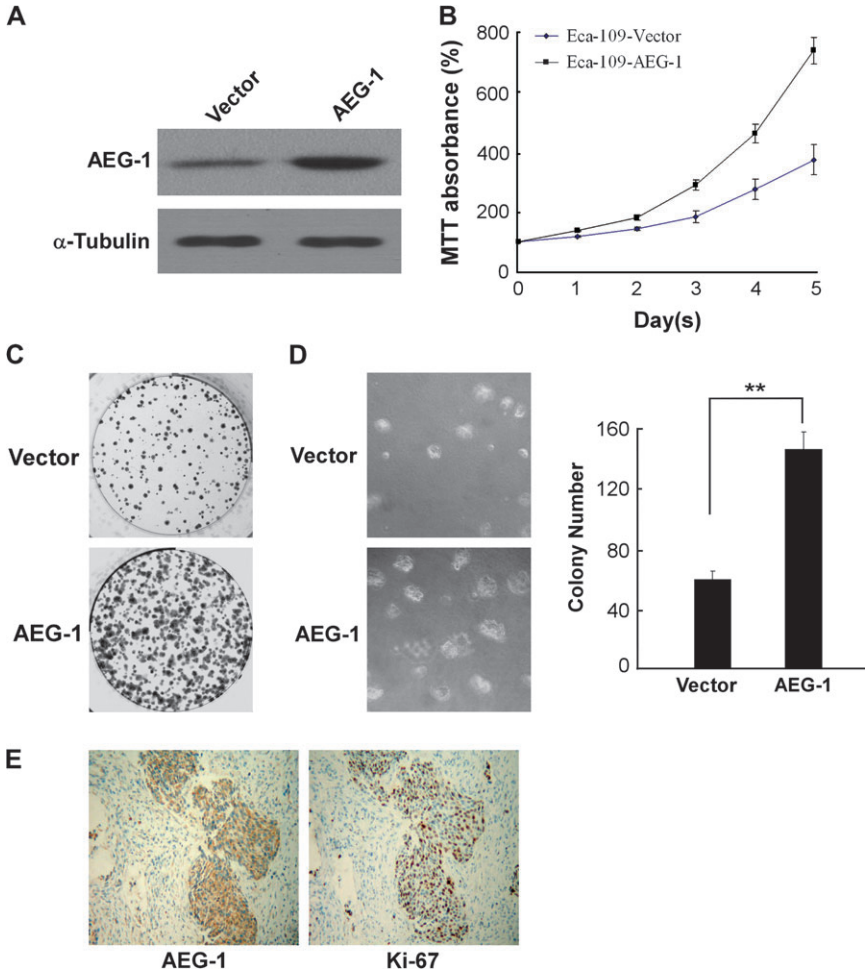

Fig. 3. Upregulation of AEG-1 enhances proliferation and tumorigenicity activity of Eca-109 cells. (**A**) Ectopic expression of AEG-1 in Eca-109 cells analyzed by western blotting. α-Tubulin was used as a loading control. (**B**) Ectopic expression of AEG-1 stimulates Eca-109 cell proliferation as determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assays. (**C**) Colony formation assay shows that the upregulation of AEG-1 promotes cell growth. (**D**) overexpression of AEG-1 promotes cell growth as determined by anchorage-independent growth ability assays. (**E**) Immunohistochemical analyses of AEG-1 and Ki67 in ESCC tissues. Error bars represent mean ± SD from three independent experiments.

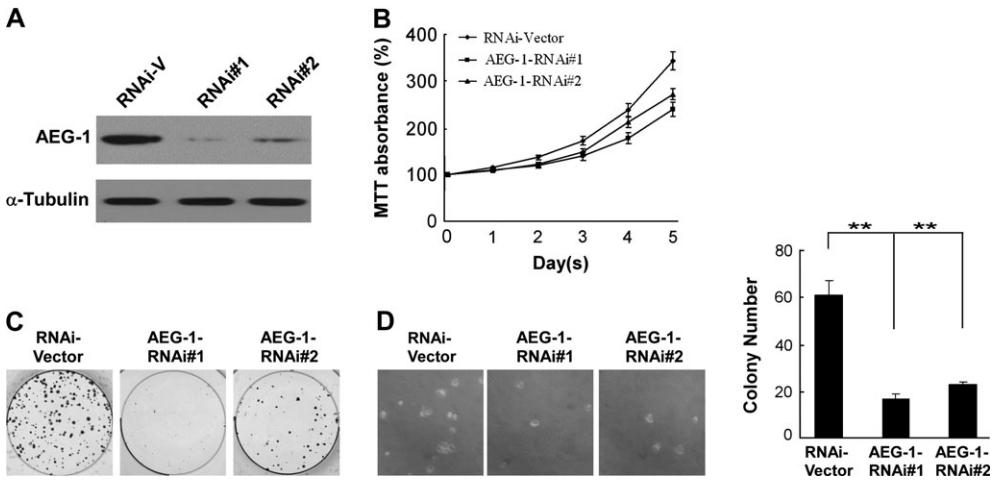

Fig. 4. Knockdown of endogenous AEG-1 inhibit cell growth and tumor-promoting activity. (**A**) Knockdown of AEG-1 in specific shRNAs-transduced stable Eca-109 cells. α-Tubulin was used as a loading control. (**B**) Silencing endogenous AEG-1 inhibits cell growth as determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assays. (**C**) Silencing endogenous AEG-1 inhibits cell growth as determined by colony formation assays. (**D**) Silencing endogenous AEG-1 inhibited cell growth as determined by anchorage-independent growth ability assays. Error bars represent mean ± SD from three independent experiments.

To further examine the role of AEG-1 expression in cancer progression, the expression patterns of AEG-1 and Ki67 were analyzed in previously mentioned 168 samples of paraffin-embedded, archived ESCC tissues. As shown in Figure 3D, areas with low levels of AEG-1 had only marginally detectable expression Ki67, whereas specimens with strong staining AEG-1 signal showed high levels of Ki67 expression. Statistical analyses indicated that the correlation between AEG-1 and Ki67 expression was significant ($P = 0.039$) (Table I), which was further confirmed by Spearman correlation analysis ($r = 0.159$, $P = 0.039$) (supplementary Table 2 is available at *Carcinogenesis* Online). These results suggest that AEG-1 is overexpressed in highly proliferative human ESCC cells, which further supports the notion that the overexpression of AEG-1 is associated with the progression of the disease.

AEG-1 regulates cell proliferation factors cyclin D1 and p27^{Kip1} though the AKT/FOXO3a pathway in ESCC cells

Since deregulation of the AEG-1 expression appears tightly linked to the proliferation of ESCC cells, we further investigated whether cell cycle factors could be regulated by AEG-1. Western blot analysis revealed that ectopic expression of AEG-1 did not affect the expression of cell cycle promoters such as CDK2, cyclin A and cyclin B (data not shown), whereas the expression of cyclin D1 is upregulated and p27^{Kip1} is downregulated in AEG-1-infected cells compared with vector-infected control cells [Figure 5A and supplementary Figure1C and D (available at *Carcinogenesis* Online)]. In contrast, significant increases in the expression of p27^{Kip1} and decreases of cyclin D1 were shown in AEG-1 knockdown ESCC cells (Figure 5B). Furthermore, real-time reverse transcription–PCR results indicated that the modulation of p27^{Kip1} and cyclin D1 by AEG-1 were regulated at the transcriptional level (Figure 5C and D).

Since it has been demonstrated that the expression of p27^{Kip1} and cyclin D1 could be transcriptionally regulated by FOXO3a and that the transcriptional activity of FOXO3a could be modulated by AKT phosphorylation, we hypothesized that upregulating AEG-1 expression may activate AKT/FOXO3a transduction signaling. As shown in Figure 5A, the phosphorylation levels of FOXO3a and AKT were indeed increased in AEG-1-infected ESCC cells. In contrast, the expression levels of phosphorylated FOXO3a and phosphorylated AKT in AEG-1 knockdown cells are much lower than in vector control cells (Figure 5B). As expected, the phosphorylation level of GSK-3β, a downstream target protein of AKT, was shown to increase in AEG-1-overexpressing cells, whereas it decreased in AEG-1-knockdown cells (Figure 5A and B). Taken together, these data indicated that AEG-1 might play an important role in the regulation of cell proliferation factors cyclin D1 and p27^{Kip1} though the AKT/FOXO3a pathway in ESCC cells.

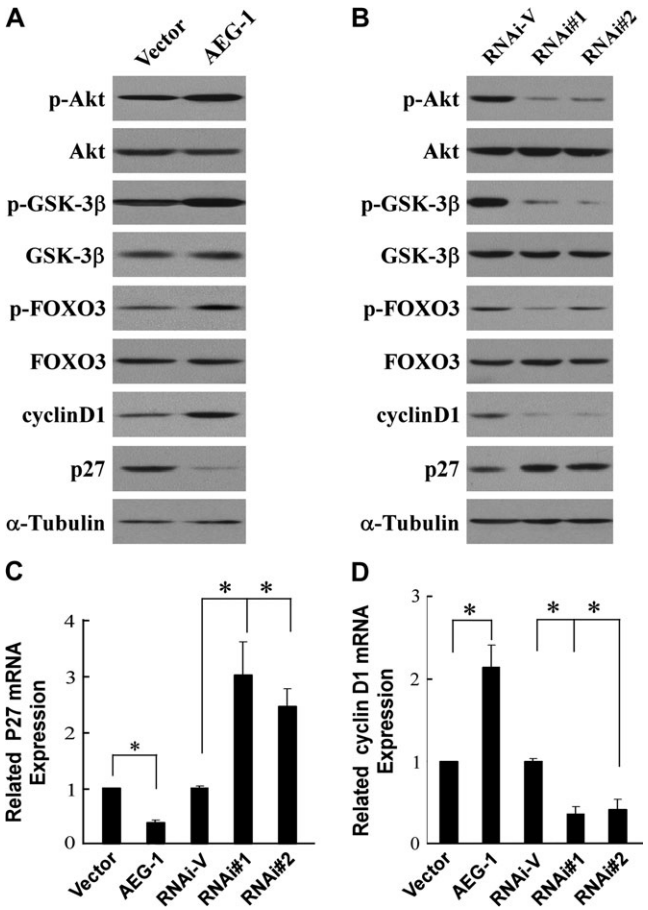

Fig. 5. AEG-1 regulates cell proliferation factors cyclin D1 and p27^{Kip1} though the AKT/FOXO3a pathway in ESCC cells. (**A**) Western blotting analysis of the expression of phosphorylated AKT, total AKT, phosphorylated GSK-3β, total GSK-3β, phosphorylated FOXO3a, total FOXO3a, cyclin D1 and p27^{Kip1} proteins in indicated vector-infected and AEG-1-infected ESCC cell lines. (**B**) Western blotting analysis of the expression of phosphorylated AKT, total AKT, phosphorylated GSK-3β, total GSK-3β, phosphorylated FOXO3a, total FOXO3a, cyclin D1 and p27^{Kip1} proteins in indicated vector-infected and AEG-1-/RNAis-infected-ESCC cell lines. (**C** and **D**) Relative mRNA expression of p27^{Kip1} (C) and cyclin D1 (D) in indicated ESCC cell lines were determined by real time reverse transcription–PCR. Error bars represent mean ± SD from three independent experiments.

Discussion

China has the highest incidence and mortality of ESCC in the world. Around 50%, all newly identified ESCC patients in the world every year are in China. The highest annual incidence rates in the most high-risk areas of China are ~161/100000 for males and 103/100000 for females. Due to the lack of effective early diagnosis biomarkers, ESCC is the most common cause of cancer-related deaths in these areas, and the average 5 years survival rate of ESCC is <10% (21–24). In the current study, we found that patients with higher AEG-1 expression had shorter overall survival time, whereas patients with lower AEG-1 expression had better survival. Multivariate analysis revealed that AEG-1 expression might be an independent prognostic indicator of the survival of ESCC patients. Our results strongly suggested that AEG-1 could be a valuable biomarker for the prediction of ESCC prognosis.

Recently, numerous reports have demonstrated that oncoprotein AEG-1 is linked to the biological processes such as cancer cell survival, apoptosis and migration. AEG-1 has been also found to be upregulated in several types of human cancers, including breast cancer, glioblastoma multiform, melanoma and prostate cancer (11,12,14,17). All these findings have implicated the overexpression of AEG-1 in the initiation and progression of cancer. To investigate whether the upregulation of AEG-1 is also related to the progression of ESCC, we performed studies to characterize the expression of AEG-1 in ESCC cell lines and clinical ESCC tissues. Our results clearly showed that AEG-1 is upregulated at both the messenger RNA and protein levels in ESCC cell lines as well as ESCC tissue. In addition, immunohistochemical analysis showed that AEG-1 was overexpressed in 156 of 168 (92.9%) ESCC specimens, whereas weak or no signal was detected in the normal esophageal tissue. Statistical analysis revealed that AEG-1 expression significantly correlates with clinical staging, tumor–node–metastasis classification and histological differentiation. Taken together, our data suggest that AEG-1 may play critical roles in the pathogenesis of ESCC.

To further investigate the biological role of AEG-1 expression in ESCC progression, we studied the gain or loss function of AEG-1 through its ectopic overexpression or an AEG-1 knockdown by short hairpin RNAi in ESCC models. The data show that a deregulation of AEG-1 in ESCC cells and NEEC cells could promote cell

proliferation due to the upregulation of cyclin D1 and the downregulation of $p27^{Kip1}$. In addition, the biological function of AEG-1 in ESCC progression was further supported by a significant correlation between AEG-1 and Ki67 expression in ESCC measured with immunohistochemical analysis. Taken together, our results not only suggest a potentially promising use of AEG-1 as a prognostic and survival indicator but also implicate a possible link between the biological function of AEG-1 and the pathogenesis of ESCC, which could eventually lead to the development of a new anti-ESCC strategy.

Numerous genetic alterations, such as amplification and overexpression of c-Myc and EGFR, as well as mutations of p53 and RB and deletion of p16INK4A, and several pathways, such as PI3K/AKT pathway and nuclear factor-kappa B pathways, have been demonstrated to be frequently linked to the development and progression of ESCC (25–30). Overexpression of AEG-1 could enhance agar cloning efficiency and increase matrigel invasion of cancer cells through increasing the nuclear factor-kappa B transcription activity by forming the basal transcription machinery with CBP and other transcriptional activators (31,32). In addition, ectopic expression of AEG-1 could prevent serum starvation from apoptosis through the activation of PI3K/AKT signaling, whereas silencing endogenous AEG-1 could inhibit prostate cancer progression through the downregulation of AKT activity (13,14). Therefore, these studies have presented new insights into the potential role of AEG-1 in the development and progression of human ESCC. Further investigation is needed of the association with the development and progression of ESCC and the activations of nuclear factor-kappa B and AKT/PI3K through the upregulation of AEG-1.

It is noteworthy that AEG-1 has been found, in our study, to strongly correlate with gender ($P = 0.042$). The best explanation may be that men are more probably to be exposed to exogenous agents, such as smoking and alcohol (33–36). A number of tobacco carcinogens, including polycyclic aromatic hydrocarbons, aromatic amines and phenol aldehydes, have been reported to be associated with ESCC (37). Alcohol consumption is another etiological factor for developing ESCC, and increased alcohol consumption could further increase the risk for ESCC among smokers (34,38).

In conclusion, our findings suggest that the upregulation of AEG-1 may be useful as a prognostic marker of ESCC progression. Further study of the molecular mechanism of AEG-1 involvement in the development and progression of ESCC is warranted. Investigation is also needed to determine whether AEG-1 could be used as a target for novel anti-ESCC therapies.

Supplementary material

Supplementary Tables 1– 3 and Figure 1 can be found at http://carcin.oxfordjournals.org/

Funding

Foundation of Ministry of Science and Technology of China (30670803, 30770836, 30771110, 30870963, 30831160517); Foundation of Ministry of Science and Technology of Guangdong Province, People's Republic of China (07001503, 8251008901000006, 2008A030201006); Municipal Science and Technology Bureau Foundation of GuangZhou (2007J1-C0271); Program for New Century Excellent Talents in University (NCET-07-0877); Foundation of Ministry of Education (200805580047, 2008890).

Acknowledgements

Conflict of Interest Statement: None declared.

References

1. Kamangar,F. *et al*. (2006) Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. *J. Clin. Oncol.*, **24**, 2137–2150.
2. Parkin,D.M. *et al*. (2001) Cancer burden in the year 2000. The global picture. *Eur. J. Cancer*, **37**, S4–S66.
3. Xing,D. *et al*. (2003) Genetic polymorphisms and susceptibility to esophageal cancer among Chinese population. *Oncol. Rep.*, **10**, 1615–1623.
4. Hiyama,T. *et al*. (2007) Genetic polymorphisms and esophageal cancer risk. *Int. J. Cancer*, **121**, 1643–1658.
5. Koshy,M. *et al*. (2004) Multiple management modalities in esophageal cancer: combined modality management approaches. *Oncologist*, **9**, 147–159.
6. Younes,M. *et al*. (2002) Incidence and survival trends of esophageal carcinoma in the United States: racial and gender differences by histological type. *Scand. J. Gastroenterol.*, **37**, 1359–1365.
7. Lin,D.C. *et al*. (2009) Protein alterations in ESCC and clinical implications. *Dis. Esophagus*, **22**, 9–20.
8. Yie,S.M. *et al*. (2007) Expression of HLA-G is associated with prognosis in esophageal squamous cell carcinoma. *Am. J. Clin. Pathol.*, **128**, 1002–1009.
9. Ashida,A. *et al*. (2006) Expression profiling of esophageal squamous cell carcinoma patients treated with definitive chemoradiotherapy: clinical implications. *Int. J. Oncol.*, **28**, 1345–1352.
10. Kang,D.C. *et al*. (2005) Cloning and characterization of HIV-1 inducible astrocyte elevated gene-1, AEG-1. *Gene*, **353**, 8–15.
11. Lee,S.G. *et al*. (2006) Astrocyte elevated gene-1 (AEG-1) is a target gene of oncogenic Ha-ras requiring phosphatidylinositol 3-kinase and c-Myc. *Proc. Natl Acad. Sci. USA*, **103**, 17390–17395.
12. Emdad,L. *et al*. (2007) Astrocyte elevated gene-1: recent insights into a novel gene involved in tumor progression, metastasis and neurodegeneration. *Pharmacol. Ther.*, **114**, 155–170.
13. Lee,S.G. *et al*. (2008) Astrocyte elevated gene-1 activates cell survival pathways through PI3K-Akt signaling. *Oncogene*, **27**, 1114–1121.
14. Kikuno,N. *et al*. (2007) Knockdown of astrocyte-elevated gene-1 inhibits prostate cancer progression through upregulation of FOXO3a activity. *Oncogene*, **26**, 7647–7655.
15. Brown,D.M. *et al*. (2004) Metadherin, a cell surface protein in breast tumors that mediates lung metastasis. *Cancer Cell*, **5**, 365–374.
16. Sarkar,D. *et al*. (2008) Molecular basis of NF-KB activation by astrocyte elevated gene-1. *Cancer Res.*, **68**, 1478–1484.
17. Li,J. *et al*. (2008) Astrocyte elevated gene-1 is a novel prognostic marker for breast cancer progression and overall patient survival. *Clin. Cancer Res.*, **14**, 3319–3326.
18. Andl,C.D. *et al*. (2003) Epidermal growth factor receptor mediates increased cell proliferation, migration, and aggregation in esophageal keratinocytes *in vitro* and *in vivo*. *J. Biol. Chem.*, **278**, 1824–1830.
19. Sobin,L.H. *et al*. (1997) *International Union Against Cancer (UICC). TNM Classification of Malignant Tumours*. 5th edn. New York, NY: Wiley. pp. 54–58.
20. Livak,K.J. *et al*. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) method. *Methods*, **25**, 402–408.
21. Parkin,D.M. *et al*. (1993) Estimates of the worldwide incidence of eighteen major cancers in 1985. *Int. J. Cancer*, **54**, 1–13.
22. An,J.Y. *et al*. (2004) Proteomic analysis of blood level of proteins before and after operation in patients with esophageal squamous cell carcinoma at high-incidence area in Henan Province. *World J. Gastroenterol.*, **10**, 3365–3368.
23. Lu,S.M. *et al*. (2008) Characterization of one newly established esophageal cancer cell line CSEC from a high-incidence area in China. *Dis. Esophagus*, **21**, 309–315.
24. Yang,L. *et al*. (2003) Time trend in cancer mortality in China, 1987–1999. *Int. J. cancer*, **106**, 771–783.
25. Mandard,A.M. *et al*. (2000) Genetic steps in the development of squamous cell carcinoma of the esophagus. *Mutat. Res.*, **462**, 335–342.
26. Lehrbach,D.M. *et al*. (2003) Molecular aspects of esophageal squamous cell carcinoma carcinogenesis. *Arq. Gastroenterol.*, **40**, 256–261.
27. Li,B.in *et al*. (2007) Id-1 activation of PI3K/Akt/NFkB signaling pathway and its significance in promoting survival of esophageal cancer cells. *Carcinogenesis*, **28**, 2313–2320.
28. Tian,F. *et al*. (2006) Nuclear factor-κB signaling pathway constitutively activated in esophageal squamous cell carcinoma cell lines and inhibition of growth of cells by small interfering RNA. *Acta Biochim. Biophys. Sin.*, **38**, 318–326.
29. Chen,L.F. *et al*. (2004) Shaping the nuclear action of NF-kappaB. *Nat. Rev. Mol. Cell Biol.*, **5**, 392–401.

30. Perkins,N.D. (2007) Integrating cell-signalling pathways with NF-kappaB and IKK function. *Nat. Rev. Mol. Cell Biol.*, **8**, 49–62.
31. Emdad,L. *et al*. (2006) Activation of the nuclear factor kappaB pathway by astrocyte elevated gene-1: implications for tumor progression and metastasis. *Cancer Res.*, **66**, 1509–1516.
32. Sarkar,D. *et al*. (2008) Molecular basis of nuclear factor-kappaB activation by astrocyte elevated gene-1. *Cancer Res.*, **68**, 1478–1484.
33. Freedman,N.D. *et al*. (2007) A prospective study of tobacco, alcohol, and the risk of esophageal and gastric cancer subtypes. *Am. J. epidemiol.*, **165**, 1424–1433.
34. Nasrollahzadeh,D. *et al*. (2008) Opium, tobacco, and alcohol use in relation to oesophageal squamous cell carcinoma in a high-risk area of Iran. *Br. J. Cancer*, **98**, 1857–1863.
35. Gao,Y.T. *et al*. (1994) Risk factor for esophageal cancer in Shanghai, China. I. Role of cigarette smoking and alcohol drinking. *Int. J. Cancer*, **58**, 192–196.
36. Fan,Y. *et al*. (2008) Alcohol, tobacco, and diet in relation to esophageal cancer: the Shanghai Cohort Study. *Nutr. Cancer*, **60**, 354–363.
37. Hecht,S.S. *et al*. (1996) Lung, and esophageal carcinogenesis. In: Aisner,J., Arriagada,R., Green,M.R., Martini,N., Perry,M.C. (eds) *Comprehensive Textbook of Thoracic Oncology.* Baltimore, MD: Williams's and Wilkins, pp. 25–50.
38. Stoner,G.D. *et al*. (2007) Chemoprevention of esophageal squamous cell carcinoma. *Toxicol. Appl. Pharmacol.*, **224**, 337–349.

Received December 20, 2008; revised March 16, 2009; accepted March 16, 2009