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Overexpression of AEG-1 in renal cell carcinoma and its correlation with tumor nuclear grade and progression

W. CHEN, Z. KE, H. SHI, S. YANG, L. WANG

Department of Pathology, the First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China 510080, e-mail: wanglt4@126.com correspondence to: L. wang, Department of Pathology, the First Affiliated Hospital, Sun Yaat-sen University, Guangzhou, China. 510080 e-mail: wanglt4@126.com Fax: 020-87331780

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The study was aimed at detecting the expression of a newly found oncogene, astrocyte elevated gene-1 (AEG-1), in renal cell carcinoma (RCC) and its correlation with histopathologic features and the survival of patients. Real-time reverse transcription-PCR and Western blot showed markedly higher expression of AEG-1 in 8 cases of RCC tissue compared with the paired normal tissue from the same patient. The expression level of AEG-1 was also increased in four RCC cell lines in contrast with normal tubular epithelial human kidney cells 2 (HK-2) at both mRNA and protein levels. Furthermore, immunohistochemistry analysis showed highly expressed AEG-1 in 96 of 102 (94.1%) cases of paraffin-embedded archival RCC tissue. Statistical analysis showed a significant correlation of AEG-1 expression with tumor grade ($P < 0.001$), clinical staging ($P = 0.003$), T classification ($P = 0.003$) as well as metastasis classification ($P=0.032$). The means for survival time of low AEG-1 expression group was 76.98m while high AEG-1expression group was 60.94m. Our results suggest that AEG-1 protein is overexpressed in RCC and plays an important role in tumor differentiation and progression. High AEG-1 expression is closely associated with poor prognosis.

Key words: Astrocyte elevated gene-1, AEG-1, renal cell carcinoma, prognosis

Renal cell carcinoma (RCC) is the most common malignancy of adult urinary tract which accounts for approximately 3% of all adult malignancies [1]. Clear-cell type (CC-RCC) and papillary RCC (PRCC) are the predominant type and comprises 80% and 10% of sporadic RCC repectively; chromophobe, collecting ducts of Bellini and renal medullary carcinoma represent the less common types [2]. Patients with advanced RCC still remain incurable due to the metastasis often resistant to conventional chemotherapy and hormonal therapies. Unfortunately, 25-30% patients have metastasis at diagnosis; and still 20-30% of the patients will develop metastasis post-nephrectomy though they have localized tumor at diagnosis [3]. Therapeutic options in these patients are limited and prognosis remains poor. Recent advances in the use of novel targeted agents particularly involved in HIF/VEGF and mTOR pathways appear to improve the prognosis of the patients [4]. Though conventional pathologic parameters such as tumor size, nuclear grade, and metastasis status are of great significance in predicting the prognosis of the patients, we still need to deepen our understanding on the molecular mechanism of the pathogenesis of RCC and to identify new additional predictors and novel therapeutic targets as well.

Astrocyte elevated Gene-1(AEG-1) is a newly cloned gene from primary human fetal astrocytes induced by HIV-1 and tumor necrosis factor-α [5]. It is very clear now that AEG-1 is far more than just a gene regulated in astrocytes which contributes centrally to human gliomas. Numerous studies confirmed that it is also over-expressed in various human cancers including breast, lung, prostate, glioblastoma multiform and esophageal cancer [6–10]. AEG-1 plays an important role in PI3K/Akt signaling pathway and nuclear factor κ-B signaling pathway which regulate fundamental cellular functions, including proliferation, growth and survival, thus have critical function in cancer cell migration and invasion [11, 12].

In this study we found that AEG-1 was markedly overexpressed in RCC cell lines and tissue of various histological types. We also found that expression of AEG-1 was correlated with the clinical staging, nuclear grade and survival of the patients. Thus, our results suggested that AEG-1 may be involved in pathogenesis of RCC and might be a new predictor for patients with RCC.

Materials and methods

Cell lines. Human kidney 2 (HK-2) which is a proximal tubular cell line derived from normal kidney was cultured in the DMEM/F12 medium supplemented with 10% fetal bovine serum (HyClone). RCC cell lines, including 786-0, OS-RC-1 were grown in 1640 medium (Invitrogen), Caki-2 in Macoy's 5A medium and ACHN in MEM medium, which all supplemented with 10% fetal bovine serum (HyClone).

Clinical background of the specimen. Eight pairs of primary RCC tissue and matched normal tissue obtained from the same patient were used for real-time RT-PCR and Westernblot. The percentages of tumor purity in sections adjacent to the regions used for RNA extraction were estimated during routine histopathologic analysis. Patients' consents and approval from the Institutional Research Ethics Committee were obtained for the use of these clinical materials for research purposes. Totally, 102 paraffin-embedded RCC samples are used, which were histopathologically and clinically diagnosed at the 1st Affiliated Hospital of Sun Yat-sen University from 2003 to 2008. Clinical stage and histopathologic classification were determined according to the WHO criteria (2004) [2]. Clinical background of the samples is summarized in Table 1.

RNA extraction, reverse transcription, and real-time PCR. Total RNA from cells and primary tumor materials was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's instruction. The extracted RNA was pretreated with RNase-free DNase, and 2μg of RNA from each sample was used for cDNA synthesis primed with random hexamers. Real-time PCR was employed to determine the fold of increase of AEG-1 mRNA in each of the tumor tissue relative to the paired normal renal tissue taken from the same patient. Expression data were normalized to the geometric mean of housekeeping gene GAPDH to control the variability in expression levels. Real-time PCR primers for AEG-1 (sense 5'AAATAGCCAGC CTATCAAGACTC3'; antisense (5'TTCAGACTTGGTCTGTGAAGGAG3') and GAPDH (sense 5'GACTCATGACCACAGTCCATGC antisense 5'AGAGGCAGGGATGATGTTCTG) were synthesized by Invitrogen (Shanghai China). Amplication reaction assays were set up triplicate for each sample using the SYBR Green (TaKaRa) system. The $2^{-\Delta\Delta Ct}$ method was used to quantify the gene expression changes [13].

Western blotting. Cells were harvested in sampling buffer [62.5 mmol/L Tris-HCl (pH 6.8), 10% glycerol, 2% SDS] and heated for 5 min at 100°C. Protein concentration was determined using Bio-Rad Protein Assay Reagent. 50ug of protein underwent SDS-PAGE and cells were transferred onto polyvinylidene difluoride membranes (Roche). The membrane was probed with a 1:1000 diluted anti-AEG-1 rabbit antibody (Abcam) and incubated overnight at 4°C. Then the membrane was washed with TBS. The second antibody HRP-conjugated mouse anti-rabbit IgG (1:1,000) was added and incubate for 2 hours at room temperature. The membrane was reacted with enhanced chemiluminescence (Pierce) according to the manufacturer's protocols and then exposed and displayed. The amount of each sample was controlled by GAPDH.

Table 1. Clinicopathologic characteristics of patient samples with renal cell carcinoma

No. of cases (%)		No. of cases (%)	
Age(years)		T Classification	
≤50	44	T1	76
>50	58	T2	21
Gender		T3	5
Male	73	N Classification	
Female	29	N0	96
Histology		N1-2	6
CCRCC	86	Distant metastasis	
Non-CCRCC*	16	M0	88
Clinical stage		M1	14
I	66	Nuclear grade **	
II	13	Grade1	16
III	7	Grade2	60
IV	16	Grade3-4	20

* 10 cases of papillary type, 3 cases of chromophobe cell type and 3 cases of collecting ducts of Bellini.

** Fuhrman 4-tiered grading system is used and applied to 86 cases of CCRCC and 10 cases of PRCC

Immunohistochemistry. Immunohistochemical analysis was done to detect AEG-1 expression in 102 human RCC tissues. In brief, paraffin-embedded specimens were cut into 4μm sections and baked at 65°C for 30 min. The sections were deparaffinized with xylenes and rehydrated. Sections were submerged into Citrate antigenic retrieval buffer and microwaved for 8min and then were treated with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity, followed by incubation with 5% bovine serum albumin to block nonspecific binding. Rabbit anti AEG-1 (1:400 Abcam) was incubated with the sections overnight at 4°C. For negative controls, the rabbit anti-AEG-1 antibody was replaced with normal goat serum. After washing, the tissue sections were treated with HRP conjugated anti-rabbit secondary antibody (DAKO) for 30 min immersed in 3.3'-diaminobenzidine and counterstained with 10% Mayer's hematoxylin, dehydrated, and mounted.

The degree of immunostaining was reviewed and scored independently by two observers, based on both the proportion of positively stained tumor cells and the intensity of staining [6]. The proportion of tumor cells was scored as follows: 0 (no positive tumor cells), 1 (<10% positive tumor cells), 2 (10-50% positive tumor cells), and 3 (>50% positive tumor cells). The intensity of staining 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). The staining index was calculated as staining intensity score proportion of positive tumor cells. Using this

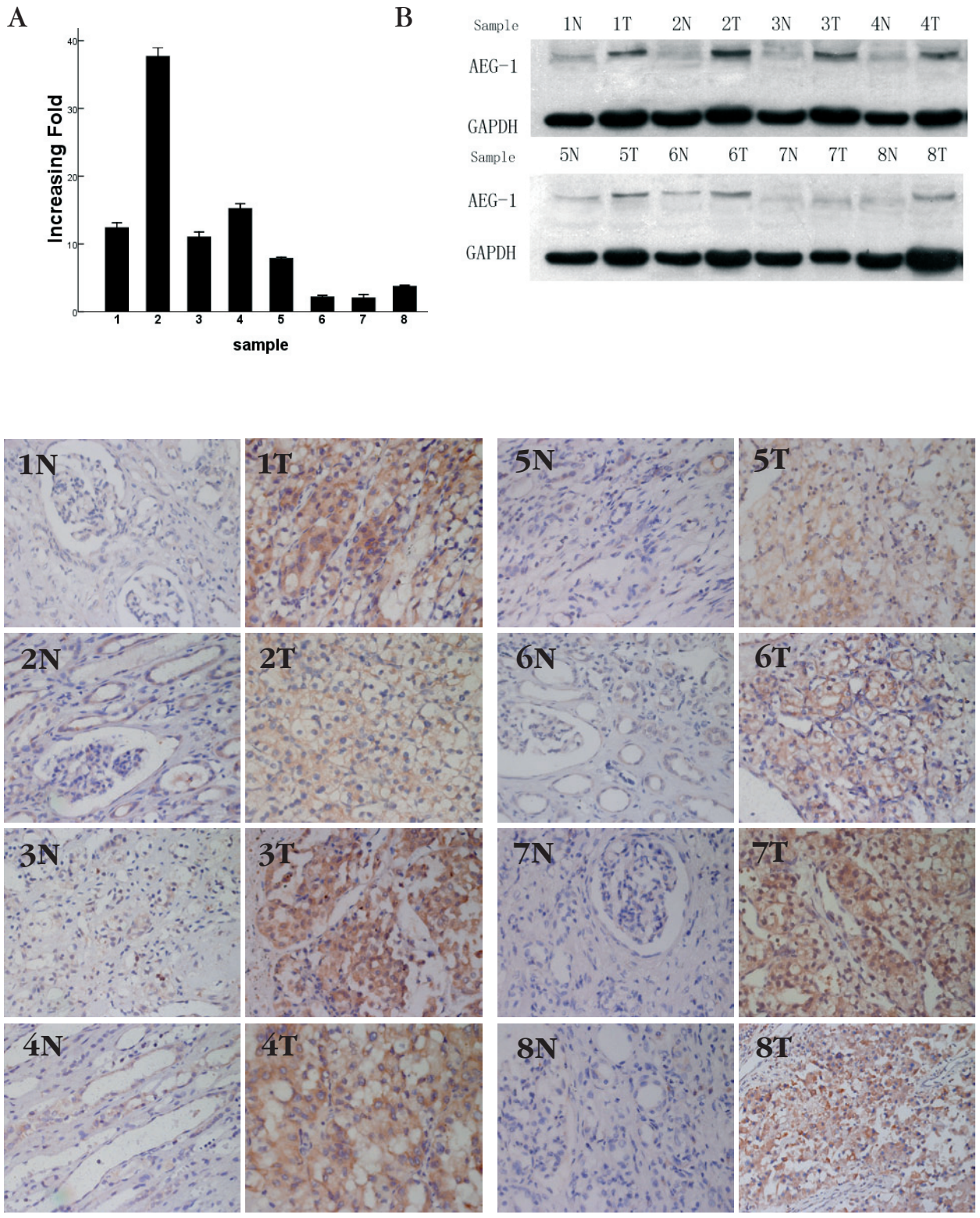

Fig. 1.Expression of AEG-1 was elevated in RCC tissue as compared with adjacent normal kidney tissue. (A) The average tumor/normal tissue ratios of AEG-1 expression were quantified by real-time RT-PCR and normalized for GAPDH. Columns, mean from three parallel experiments; bars, standard deviation (B) Expression of AEG-1 protein in each of the tumors and adjacent normal tissue paired from the same patient by westernblot. (C) IHC showed that AEG-1protein increased in the RCC tissue compared to the paired adjacent normal kidney tissue from the same patient which was consistent with westernblot.

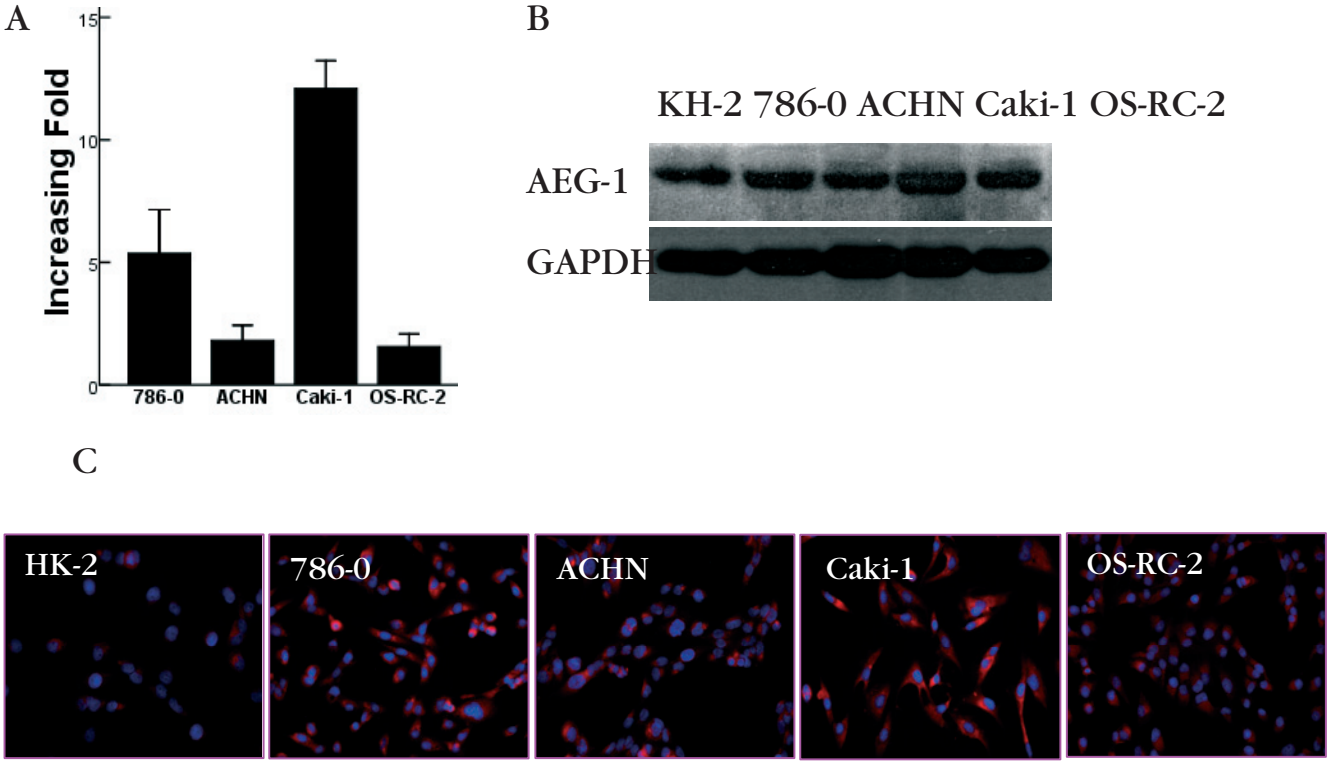

Fig. 2. The expression of AEG-1 protein and mRNA was elevated in RCC cell lines as compared with that in normal human kidney tubular epithelial cell HK-2. (A) The average ratios of AEG-1 expression quantified by real-time RT-PCR in HK-2 and RCC cell lines (786-0, ACHN, Caki-1, OS-RC-2). Columns, mean from three parallel experiments; bars, standard deviation (B) Westernblot analysis of AEG-1 protein expression in each of the tumor cell lines and HK-2. (C) Immunoflurescence showed that AEG-1protein was increased in the renal cell carcinoma tissue compared to HK-2.

method of assessment, we evaluated the expression of AEG-1 in normal tubular epithelium and malignant lesions by determining the staining index, which scores as 0, 1, 2, 3, 4, 6, and 9. An optimal cutoff value was determined by log-rank test statistical analysis with respect to overall survival: the staining index score of ≥4 was used to define tumors as high AEG-1 expression and ≤3 as low expression of AEG-1.

*Statistical analysis***.** All statistical analyses were carried out using the SPSS 13.0 statistical software package. Chi-square test and Mann-Whitney U test was used to analyze the correlation between AEG-1 expression and the clinicopathologic characteristics. Survival curves were plotted using the Kaplan-Meier method. $P < 0.05$ in all cases was considered statistically significant.

Results

*Increased expression of AEG-1 in primary renal cell carcinoma tissue.*We examined the AEG-1 mRNA and protein expression of 8 pairs in matched normal kidney tissue and RCC tissue among which 7 cases were clear cell RCC and one was multilocular cystic RCC. Real-time RT-PCR showed that AEG-1 mRNA was up-regulated in tumor samples compared with the normal kidney tissue. The tumor/normal ratio of AEG-1 varied from approximately 2-fold to 38-fold in the eight pairs (Fig. 1A). Westernbloting analysis revealed significantly over expressed AEG-1 in RCC tissues compared with the paired normal tissue from the same patient (Fig. 1B). The results were further confirmed by immunohistochemical staining of AEG-1 in the histopathological sections of the eight cases (Fig. 1C). These findings revealed that AEG-1 was highly expressed in cancer lesions of RCC when compared with values in normal renal tissues.

Overexpression of AEG-1 in renal cell carcinoma cell lines. Real-time reverse transcription-PCR was done to detect AEG-1 mRNA level in four renal cell carcinoma cell lines compared with HK-2. As shown in Fig.2A, the results showed that the four RCC cell lines showed significantly higher levels of AEG-1 mRNA in comparison with the HK-2 cells with a increasing varying from 2 to 12-fold. In parallel with up-regulation of the AEG-1 mRNA, western blot analysis showed that the protein level was also highly expressed in tumor cell lines though it was also detected in HK-2 with a relatively low level (Fig. 1B). We notified that Caki-1, which is derived from skin metasta-

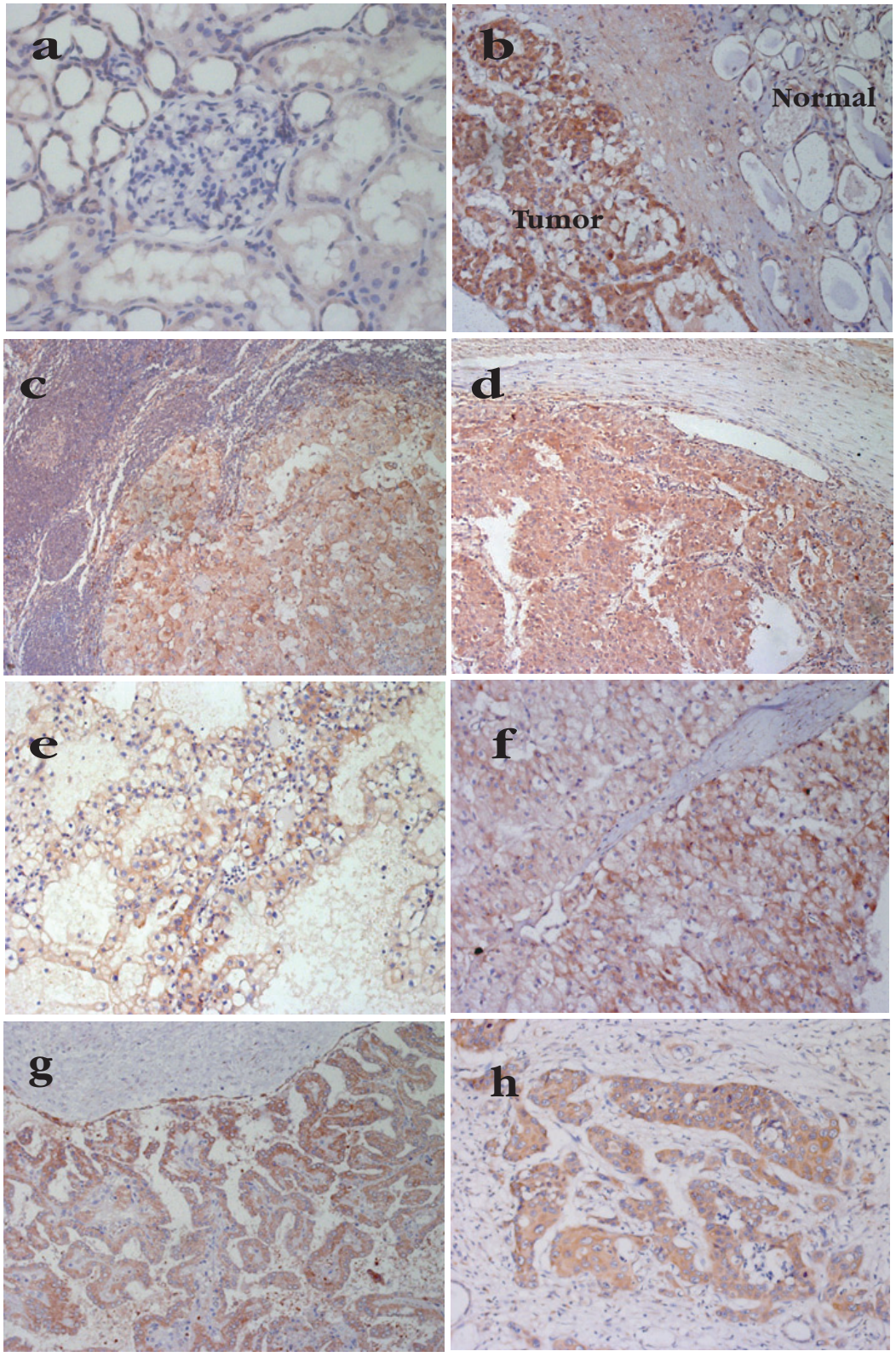

Fig. 3. AEG-1 protein was overexpressed in RCC sections as examined by immunohistochemistry. a. AEG-1 was weakly expressed in tubular epithelial cells in normal kidney. b, AEG-1 was highly expressed in the cytoplasm of the tumor cells. c and d, AEG-1 was highly expressed in lymph node metastases and neoplastic embolus in renal veins compared with the primary tumor. e, f, g h high expression of AEG-1 in CCRCC, chromophobe RCC, PRCC, and carcinoma of the collecting ducts of Bellini.(200×).

sis of CCRCC, showed both the highest mRNA and protein level of AEG-1. The result was consistent with the following immnoflurescence staining as showed in Fig. 2C. AEG-1 is highly expressed in cytoplasm of all tumor cell lines.

Overexpression of AEG-1 in archived renal cell carcinoma. For immunohistochemistry, we examined formalin-fixed, paraffin-embedded archived tumor tissues obtained from 102 patients with sporadic RCC. These cases consisted of four pathological types including 86 cases of clear cell type, 10 cases of papillary type, 3 cases of chromophobe cell type and 3 cases of collecting ducts of Bellini. All of the primary tumor as well as 6 cases of matched lymph node metastases and 7 cases of neoplastic embolus in renal vein were stained with an antibody against human AEG-1. Totally, AEG-1 was detected in 96 of 102(94.1%) cases. High expression of AEG-1 was found in 81 of 86 CCRCC, 9 of 10 PRCC, 3 of 3 chomoprobe and 3 of 3 collecting duct of Bellin RCC, as shown in Figure 3 and Table 2. Compared with the strong positivity of the tumor, AEG-1 was expressed weakly in the tubular epithelium and negative in the glomeruli in the adjacent normal kidney. Both the primary cancer and the metastases showed cytoplasmic staining. We found more intense AEG-1 staining in the lymph node metastases and in the neoplastic embolus in the renal vein, compared with the values in primary tumor. Another interesting found was the increased staining intensity of AEG-1 with the nuclear grade of the CCRCC as well as PRCC and demonstrate the strongest staining in areas of spindle-shaped cells resembling sarcomas (Fig.4). These results showed that AEG-1 was highly expressed and associated with the development and progression of RCC.

Statistical analyses. Statistical analyses were done to examine the correlation between AEG-1 expression detected by immunohistochemical staining and the cliniclpathological characteristics of renal cell carcinoma. The results were shown in table 2. There was no correlation between expression level of AEG-1 and patient age as well as gender. The expression level of AEG-1 strongly correlated with the nuclear grade of clear cell type and papillary type of RCC indicating that AEG-1 was correlated with differentiation of RCC. Poorly differentiated tumors have higher expression level of AEG-1 and cases with sarcomatoid area had the highest expression level. In nuclear grade I group, there were only 2 of 16 have high expression of AEG-1, while in nuclear 3 to 4, 16 of 20 cases having high AEG-1 expression level. We also found significant differences in AEG-1 expression in patients categorized according to the clinic stage (P=0.026), T classification (P=0.013) and M (P=0.032) which means that higher AEG-1 expression was correlated with advanced clinical stage and T classification. On the contrary, we found no significant correlation between AEG-1 expression and N classification (P=0.457).

The log-rank test showed that survival time was significantly different between the low and high AEG-1 expression groups (P = 0.002). As shown in Fig. 5, the cumulative 5-year survival rate was 91.3% in the low AEG-1 expression group, whereas it was only 52.4% in the high AEG-1 expression group. The means for survival time of low AEG-1 expression group was 76.98m (95% confidence interval, 72.94-81.02), while high AEG-1expression group was 60.94m (95% confidence interval, 53.83-68.06) indicating that higher levels of AEG-1 were associated with shorter survival time.

Table 2. Correlation between AEG-1 expression and the clinicopathological characteristics of RCC

	AEG-1 expression		
Characteristics	**Low or none**	**High**	**p value**
Age(years)			
≤50	26	18	0.052
>50	23	35	
Gender			
Male	34	39	0.639
Female	15	14	
Histology			
CCRCC	45	41	0.045
Non-CCRCC	4	12	
Clinical stage			
I	39	27	0.003
II	4	9	
III	2	5	
IV	4	12	
T Classification			
T1	43	33	0.003
T2	5	16	
T3	1	4	
N Classification			
N0	47	49	0.457
N1-2	2	4	
Distant metastasis			
M0	46	42	0.032
M1	3	11	
Nuclear grade			
Grade1	14	2	<0.001
Grade2	30	30	
Grade3-4	4	16	

Discussion

Recently, numerous studies have demonstrated that AEG-1 is upregulated and correlated with the progression and prognosis of the patients [6–8]. In order to investigate whether AEG-1 also plays a role in RCC, we performed the current study. Our data proved that AEG-1 expression was increased in both mRNA and protein levels in RCC whereas only very low AEG-1 was detected in the adjacent normal kidney tubular epithelial cells. Immunohistochemical staining showed that AEG-1 expression was increased in four classifications of RCC and AEG-1 expression level correlated with clinical stage (T classification and M classification). In clear cell type and papillary type, which are the two most

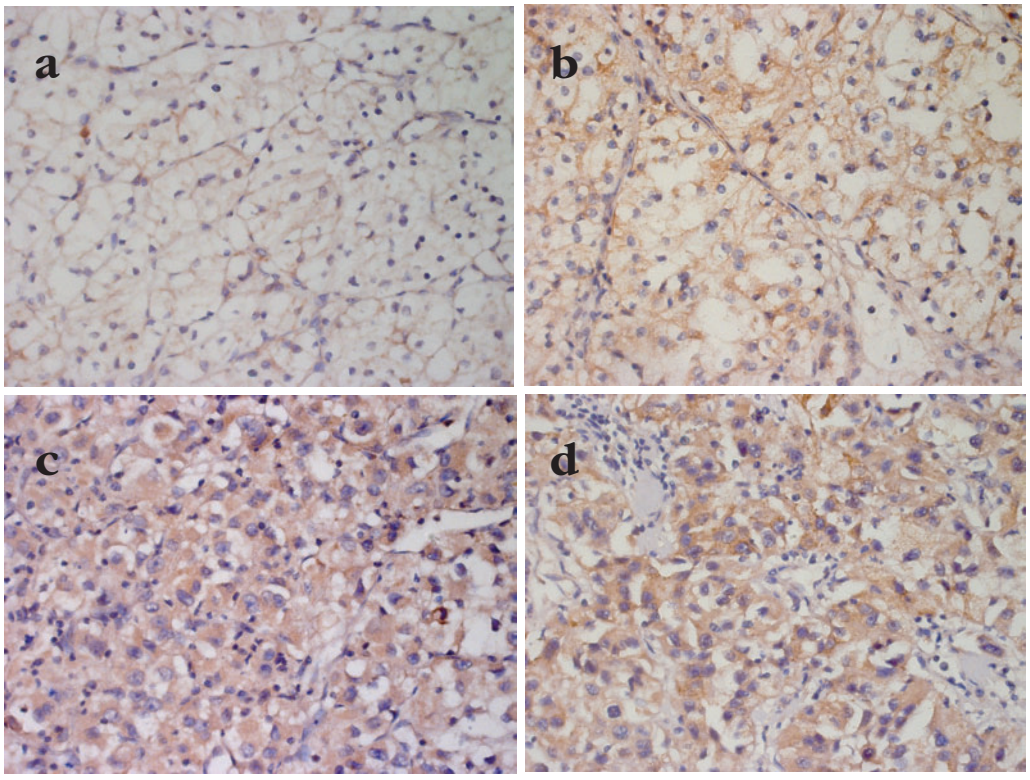

Fig. 4. AEG-1 protein expression was increased with the nuclear grade in clear cell renal cell carcinoma. a, nuclear grade 1; b, nuclear grade 2; c, nuclear grade 3; d, nuclear grade 4 with areas resembling sarcomas. (400×)

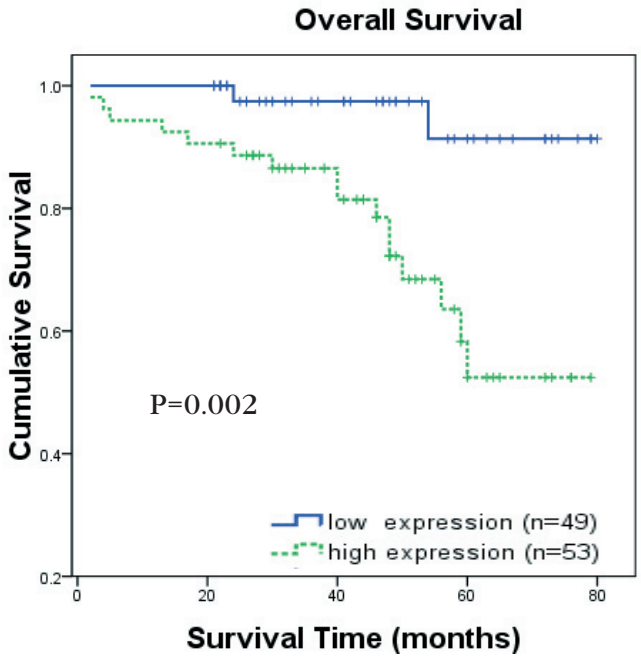

Fig. 5. Kaplan-Meier curves with univariate analyses (log-rank) of low AEG-1expression versus high AEG-1 expression in RCC. The cumulative 5-year survival rate was only 52.4% in high AEG-1 expression group (n=53; dotted line) compared with 91.3% in low AEG-1 expression group (n=49; thick line).

common type of RCC, AEG-1 expression increased with the tumor nuclear grade. Survival analysis showed that patients with higher expression of AEG-1 had significantly lower cumulative 5- year survival rate compared with patients with low AEG-1 expression. Taken together, our results indicate that AEG-1 may play important roles in the pathogenesis of RCC.

An increasing evidence shows that AEG-1-expressing tumors have increased microvessel density (MVD) and overexpression of it promotes angiogenesis both in vitro and in vivo which suggests AEG-1 plays a dominant positive role in angiogenesis thus contribute to tumor metastasis [14].In this study, we have shown that both the mRNA and protein levels of AEG-1 were markedly higher in RCC cell lines than those in human normal tubular epithelial cell HK-2. It is very interesting that in ACHN and OS-RC-2 cell lines, which show epithelial-like phenotype, real-time PCR analysis have shown only about 2-fold increase of AEG-1 message. While in 786-0 and caki-1, both of which show fibroblastic phenotype, we detected more than 6-fold increase especially in caki-1, which is a CCRCC cell line derived from skin metastasis, whose expression level of AEG-1 is as high as 12-fold compared with HK-2. In immunohistochemical analysis, we found higher expression of AEG-1 in the lymph node metastases and in the neoplastic embolus of RCC compared with the primary tumor. The highest expression of AEG-1 in Caki-1 together with the relatively higher expression in lymph node metastases and neoplastic embolus of RCC compared with the primary tumor, may indicate

that high expression of AEG-1 contribute to the metastasis in RCC though further study should be done to confirm it.

We should notify that statistical analysis of the relationship between AEG-1 staining and the clinical characteristics of patients showed a significant correlation of AEG-1 expression with the clinical staging as well as T, and M classification, while no correlation was found with N classification. This finding was inconsistent with those in other study such as breast cancer and non-small cell lung cancer[6, 8]. The most possible reason is that there is much less patients developed lymph node metastasis in our set of patients because RCC tend to metastasis by blood vessel rather than by lymph node.

Nuclear grade is the most important prognostic feature of RCC which have been validated in numerous studies [15–17]. Fuhrman 4-tiered grading system is applied to 86 cases of CCRCC and 10 cases of PRCC, which are the most common types. We found AEG-1 expression was significantly correlated with the nuclear grade ($P<0.001$) which strongly suggested that AEG-1 might be important in differentiation of RCC. Moreover, survival analysis also showed that patients in an AEG-1 high expression group revealed a 52.4% cumulative 5-yrear survival rate, which was significantly lower than that in AEG-1 low expression patients (91.3%), suggesting that AEG-1 can server as an additional predictor as nuclear grade for patient prognosis and survival.

This is the first study aimed at evaluating AEG-1 expression and its role in RCC. Our data support that AEG-1 plays a role in carcinogenesis and progression of RCC. Statistical analysis suggests that it is possible to use AEG-1 as a clinically relevant indicator for disease progression and predictor for patient survival. The PI3K/Akt pathway has a pivotal role in renal cell carcinoma pathogenesis [18] and contribute centrally to the AEG-1 induced angiogenesis [14], thus, AEG-1 may represents an ideal target for therapeutic intervention against RCC. For this purpose, further investigations clarifying its role and the mechanism in carcinogenesis and progression by functional analysis are needed.

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