

Original Article

The expression of AEG-1 and Cyclin D1 in human bladder urothelial carcinoma and their clinicopathological significance

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Received September 12, 2015; Accepted November 8, 2015; Epub November 15, 2015; Published November 30, 2015

Abstract: Objective: Astrocyte elevated gene-1 (AEG-1) and Cyclin D1 is associated with tumorigenesis and progression. The aim of this study is to investigate the expression of AEG-1 and Cyclin D1 in human bladder urothelial carcinoma (BUC) and explore their clinical and pathological significance. Methods: The expression of AEG-1 and Cyclin D1 protein were detected in 85 cases of human BUC and 16 cases of tumor-adjacent tissues by the immunohistochemical method. Results: The positive expression level of AEG-1 was 61.2% in human BUC which was higher than that in tumor-adjacent tissues (18.8%), $P=0.002$. The high expression of AEG-1 protein was correlated with the recurrence group ($P=0.004$). The positive rate of AEG-1 protein in superficial carcinoma group was lower than that of invasive cancer group ($P=0.014$). The positive expression level of Cyclin D1 was 56.5% in BUC, which was higher than that in tumor-adjacent tissues (12.5%). $P=0.001$. The high expression of Cyclin D1 protein was correlated with the recurrence group ($P=0.024$). The positive rate of Cyclin D1 protein in low grade group was lower than that of high grade group ($P=0.001$). The positive rate of Cyclin D1 protein in superficial carcinoma group was lower than that of invasive carcinoma group, ($P=0.012$). AEG-1 protein was positively correlated with Cyclin D1 protein ($r=0.567$, $P<0.001$). The log-rank test statistical analysis suggested that patients with higher AEG-1 or Cyclin D1 expression had shorter overall survival time, while patients with lower AEG-1 or Cyclin D1 expression had better survival. Conclusion: Overexpression of AEG-1 and Cyclin D1 are markedly correlated with TNM stage and recurrence of BUC. Cyclin D1 are markedly correlated with grade of BUC. Detection of AEG-1 and Cyclin D1 may be helpful to evaluate prognosis and infiltrative capability of BUC.

Keywords: Bladder, carcinoma, AEG-1, Cyclin D1, immunohistochemistry

Introduction

Bladder cancer ranks fifth among the most common cancer in Western countries and is the highest cause of death among urinary malignancies in China [1]. Its most common pathological type is urothelial carcinoma [2]. The incidence of recurrence is very high after therapy for BUC patients, which is about 50-70%. Once it recurs, it may develop to advanced pathological grade and clinical stage [3]. Cystoscopy is the most common method that has been used in the diagnosis of bladder cancer and for the follow-up of these patients. However, patients will suffer a lot with cystos-

copy. Recently, a variety of studies have focused on developing new non-invasive methods that can increase the detection rate of BUC and monitor its prognosis [4].

AEG-1, also is named metadherin (MTDH) and lysine-rich CECAM-1 co-isolated protein (LYRIC protein), was firstly cloned in the original embryos astrocytes in 2002, located in human chromosome 8 (8 q22) [5, 6]. Further research shows that AEG-1 gene is high expressed in many kinds of malignant tumors (such as cervical cancer, tongue carcinoma, gastric cancer, rectal cancer, etc.), which is closely related with the occurrence and development of malignant

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tumor [7-10]. Tumor cell cycle anomalies may lead to uncontrollable cell growth. Cyclin D1, one of the cell cycle regulatory factor, promotes cells through G1/S phase limit points. Excessive expression of Cyclin D1 gene can shorten the G1 phase, then leading to cell proliferation uncontrollable and tumor occurrence [11, 12].

In this study, we used immunohistochemical method to assay the expression of the AEG-1 and Cyclin D1 in 85 cases of bladder cancer, which was aimed at exploring their clinical and pathological significance. They may be helpful to increase the detection rate of BUC and development of a convenient diagnostic and prognosis tool monitoring of bladder cancer.

Materials and methods

Patients and tissue samples

The study protocol was approved by the ethics committee of the First People's Hospital of Taicang, and all tissue samples were collected from patients with appropriate informed consent. 85 patients underwent surgery (transurethral resection of bladder tumor, partial resection of bladder and total bladder resection) from January 2010 to January 2015. 16 cases of tumor-adjacent tissues were taken as the control group. All the patients ranged between age 36 and 88 years and their average age was 69.1 years. All patients were reviewed with the urinary system ultrasound or cystoscopy examination every 3 months after surgery. They were followed up for 3 to 60 months. None of these patients received pre-operative chemotherapy or radiotherapy. All cases were confirmed as human BUC by pathologists. Tumor-node-metastasis (TNM) stages were classified according to the 2010 Union for International Cancer Control (UICC) and the pathological grading were classified according to the 2004 World Health Organization [13, 14].

Immunohistochemical (IHC) analysis

IHC analysis was performed similarly as previously reported [15]. In brief, paraffin-embedded specimens were cut into 4- μ m sections and baked at 65°C for 30 min. The sections were deparaffinized with xylenes and rehydrated, then washed them for 3 times \times 5 min with phosphate buffer saline (PBS). 3% of the H₂O₂ incubate 10 min at room temperature. Each

section was placed into the pressure cooker filled with citric acid buffer solution and heated it to boiling, and closed the exhaust valve. When the pressure reached 0.14 MP, time 1 min, and then cooled it 60 min. PBS buffer solution washed it 3 times \times 5 min 1% bovine serum albumin (Gibco Life Technologies, Rockville, MD, USA) was used to block nonspecific binding. Rabbit anti-AEG-1 (1:200; Zymed Laboratories Inc; San Francisco, CA USA) or rabbit anti-Cyclin D1 (1:100; Wuhan Boster Biological Technology, Ltd.) was incubated with the sections overnight at 4°C. After washing, the tissue sections were treated with biotinylated anti-rabbit secondary antibody (1:250; Zymed Laboratories Inc; San Francisco, CA USA), followed by further incubation with streptavidin-horseradish peroxidase complex (Zymed Laboratories Inc; San Francisco, CA USA). Drop the freshly prepared. 100 μ L DAB solution to each section (protect from light), and observe under the microscope. Observe the staining timely. Hematoxylin stains 2 min and wash under the running water to colorless. Each section dehydrated with the alcohol, and xylene transparently process. The rabbit anti-AEG-1 antibody or rabbit anti-Cyclin D1 antibody was replaced with normal non-immune serum, as negative controls.

Evaluation of AEG-1 and Cyclin D1 staining

The slides were examined by two pathologists and the sections were evaluated according to the immunohistochemical scores (IHS) [16]. The proportion of positive cells were scored as 0 (no positive tumor cells), 1 (<10% positive tumor cells), 2 (10-50% positive tumor cells) and 3 (>50% positive tumor cells). The staining intensity was scored as 0, no staining; 1, light yellow; 2, yellow brown; and 3, brown. The final AEG-1 and Cyclin D1 staining score was calculated using the percent of staining intensity score positive tumor cell score. In the study, the AEG-1 and Cyclin D1 expression is defined as positive (+, high expression) when the score is more than 3, and negative (-, low expression) when score is less than or equal to 3.

Statistical analysis

The results were expressed as mean \pm standard deviation (S.D). SAS 9.2 and the GraphPad Prism version 5.0 software package (GraphPad Software, San Diego, CA, USA) were used for

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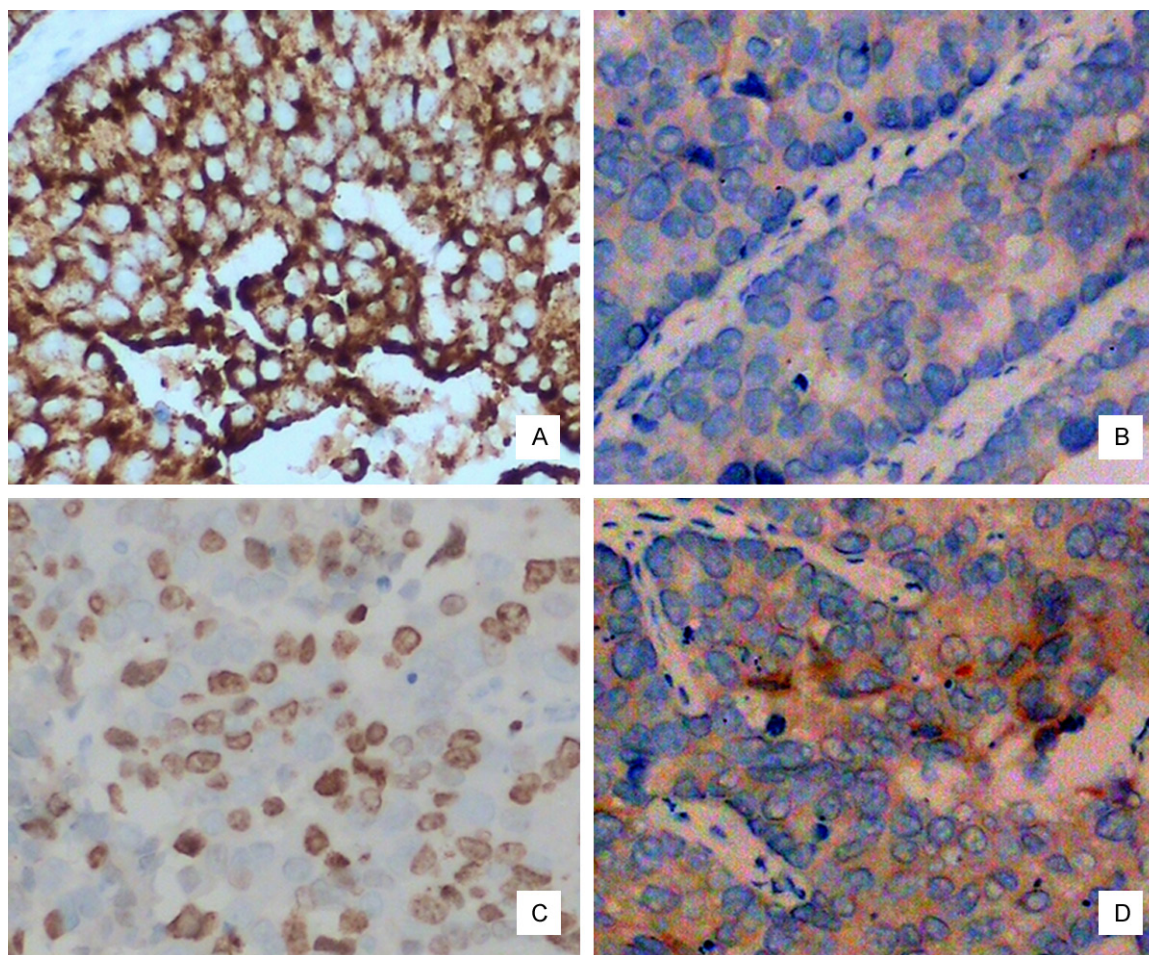


Figure 1. Immunohistochemical expression analysis of AEG-1 and Cyclin D1 protein in BUC tissue (200 \times). A. AEG-1 is positive staining in high grade of BUC tissue and its cytoplasm is stained brown. B. AEG-1 is negative staining in high grade of BUC tissue and its cytoplasm staining is weak. C. Cyclin D1 is positive staining in high grade of BUC tissue and its nucleus is stained brown. D. Cyclin D1 is negative staining in high grade of BUC tissue and its nucleus is not stained.

Table 1. Expressions of AEG-1a and Cyclin D1 in BUC and tumor-adjacent tissue

Tissue type	AEG-1 expression ^a		Cyclin D1 expression ^a	
	Negative (-)	Positive (+)	Negative (-)	Positive (+)
BUC tissue	33 (38.8)	52 (61.2)	37 (43.5)	48 (56.5)
Tumor-adjacent tissue	13 (81.2)	3 (18.8)	14 (87.5)	2 (12.5)
<i>P</i> value ^b	0.002		0.001	

^aAEG-1 and Cyclin D1 protein expression, positive means IHS are 4-9, and negative means IHS are 0-3. ^b*P* values were evaluated by chi-square test.

statistical analysis. The relationship between AEG-1 and Cyclin D1 expression and clinical parameters was evaluated using Pearson χ^2 test (SAS 9.2). The overall survival rates were calculated by the Kaplan-Meier method, and the difference in survival was compared with the log-rank test (GraphPad Prism). Differences

were considered statistically significant when $P < 0.05$.

Results

Expression of AEG-1 and Cyclin D1 protein in cancer and tumor-adjacent tissues

As was shown in **Figure 1**, AEG-1 staining was predominantly observed on the cytoplasm of tumor cells (**Figure 1A**). Cyclin D1 staining was predominantly observed on the nucleus of tumor cells (**Figure 1C**). The expression level of AEG-1 in the tumor tissues was significantly increased compared with tumor-adjacent tissues ($P = 0.002$; **Table 1**). The expression level of Cyclin D1 in the tumor tissues was sig-

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Table 2. Analysis of AEG-1 and Cyclin D1 positive expression and related factors

Related Factor	n	AEG-1 expression ^a		P value ^b	Cyclin D1 expression ^a		P value ^b
		Negative (-)	Positive (+)		Negative (-)	Positive (+)	
Age							
>65	58	21 (26.2)	37 (63.8)	0.46	23 (39.7)	35 (60.3)	0.291
≤65	27	12 (44.4)	15 (55.6)		14 (51.9)	13 (48.1)	
Gender							
Male	66	26 (39.4)	40 (60.6)	0.459	27 (40.9)	39 (59.1)	0.364
Female	19	7 (36.8)	12 (63.2)		10 (52.6)	9 (47.4)	
TNM stage							
Superficial	59	28 (47.5)	31 (52.5)	0.014	31 (52.5)	28 (47.5)	0.012
Invasive	26	5 (19.2)	21 (80.8)		6 (23.1)	20 (76.9)	
Recurrence							
Negative	48	25 (52.1)	23 (47.9)	0.004	26 (54.2)	22 (45.8)	0.024
Positive	37	8 (21.6)	29 (78.4)		11 (29.7)	26 (70.3)	
Grade							
Low	32	15 (46.9)	17 (53.1)	0.237	21 (65.6)	11 (34.4)	0.001
High	53	18 (34.0)	35 (66.0)		16 (30.2)	37 (69.8)	

^aAEG-1 and Cyclin D1 protein expression, positive means IHS are 4-9, and negative means IHS are 0-3. ^bP values were evaluated by chi-square test.

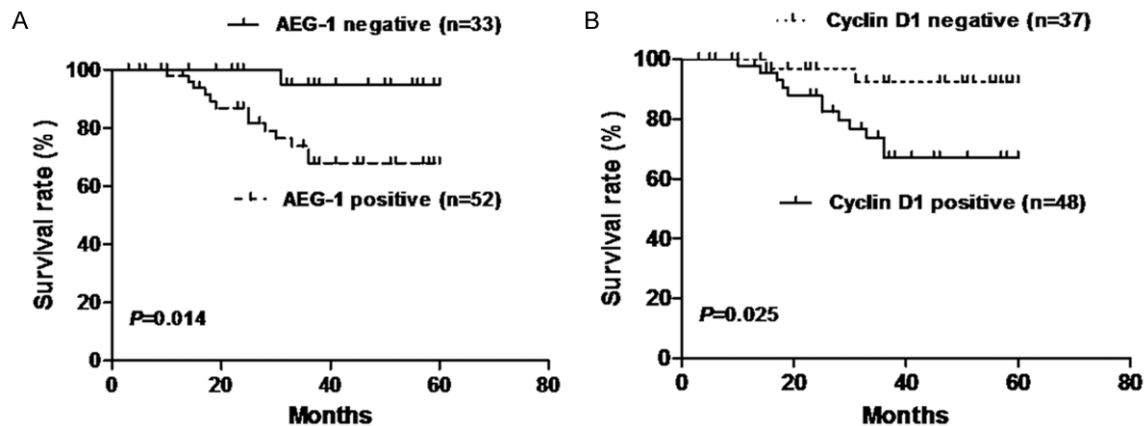


Figure 2. Kaplan-Meier survival curves of BUC patients based on AEG-1 expression (A) or Cyclin D1 expression (B).

nificantly increased compared with tumor-adjacent tissues ($P=0.001$; **Table 1**).

Relationship of AEG-1 and Cyclin D1 expression and clinicopathological parameters

AEG-1 and Cyclin D1 protein expression and clinicopathological features of BUC were examined as was shown in **Table 2**. High levels of AEG-1 protein expression were significantly correlated with tumor TNM stage and recurrence ($P=0.014$ and $P=0.004$, respectively). However, AEG-1 protein expression was not associated with gender, tumor grade and age. High levels

of Cyclin D1 protein expression were significantly correlated with tumor TNM stage, grade and Recurrence ($P=0.012$, $P=0.001$ and $P=0.024$, respectively). However, Cyclin D1 protein expression was not associated with gender and age.

Correlations between expressions of AEG-1 and Cyclin D1 and survival

The correlations were shown in **Figure 2**. Kaplan-Meier survival curves of BUC patients based on AEG-1 or Cyclin D1 expression. Patients with high AEG-1 expression showed

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Table 3. Correlations between AEG-1 and Cyclin D1 expression in BUC tissue

Cyclin D1	AEG-1		Pearson correlation coefficient (r)	χ^2	P
	+	-			
+	41	7	0.567	27.280	<0.001
-	11	26			

significantly worse survival compared with those patients with low expression ($P=0.014$, log-rank test) (**Figure 2A**). Patients with high Cyclin D1 expression showed significantly worse survival compared with those patients with low Cyclin D1 expression ($P=0.025$, log-rank test) (**Figure 2B**).

Correlations between AEG-1 and Cyclin D1 expression in BUC tissue and clinicopathological parameters

In bladder cancer tissues, the expression of AEG-1 was positively correlated with the expression of Cyclin D1 protein ($r=0.567$, $P<0.001$), **Table 3**.

Discussion

Studies have shown that AEG-1 can promote the development of tumor through various pathways. Firstly, AEG-1 can synergize with Ras gene to regulate the downstream genes of PI3K/Akt signal pathway, such as FOXO1 (Forkhead box O1), the expression of P53, AP-1 and so on, leading to significantly enhancing the proliferation and apoptosis resistance of tumor cells [17]. In addition, the high expression of AEG-1 can also activate the NF- κ B (nuclear factor kappa B) signal pathway. Within the nucleus, AEG-1 mediated the binding of p65 and transcription cofactors CBP (CSK-binding protein), and then adjust the transcription of NF- κ B target genes. NF- κ B as a kind of important transcription factors in the nuclei can regulate the expression of protein or gene like cell adhesion molecules, Cyclin D1, metal matrix protein-9, ring oxygenase-2, further regulating the proliferation, apoptosis, transfer and other links of tumor cells [18]. In addition, AEG-1 mediates tumor angiogenesis. Emdad has found that when AEG-1 overexpressed, the molecular markers (matrix metalloproteinases angiogenin-1, 2, hypoxia inducing factor alpha 1) of tumor angiogenesis correspondingly highly expressed, and when the expression of AEG-1

protein lowered, angiogenesis also reduced [19]. Long has also found that AEG-1 gene can promote the angiogenesis of cervical cancer [20].

In this study, the positive rate of AEG-1 protein in BUC is significantly higher than the tumor-adjacent tissue, suggesting that AEG-1 may participate in BUC tumorigenesis. However, AEG-1 expression levels are not significantly correlated with pathological grading in BUC patients, which may due to the relatively small sample size. In addition, the positive rate of AEG-1 protein has no relationship with the age, gender and tumor number of patients; the positive rate of AEG-1 protein in invasive carcinoma, which is much higher than superficial carcinoma, is closely related to the TNM stage, suggesting that AEG-1 play a role in the progression of BUC. The previous research found that the patients with high AEG-1 expression had short survival time in cervical cancer and tongue cancer [7, 8]. By contrast, the treatment effect and survival time of the patients with lower expression were significantly increased [18, 21]. Therefore, AEG-1 is expected to be an independent tumor prognostic factor. Our follow-up results also show that the patients with high AEG-1 protein had higher recurrence rate of tumor and worse survival compared with those patients with low AEG-1 expression. With more studies about mechanism of AEG-1 and tumor proliferation, apoptosis, and malignant progress, AEG-1 as the target drugs of gene therapy also get more and more attention. AEG-1 gene silenced by using RNA interference can reduce the proliferation and invasion ability of tumor cells [7, 22].

In combination with the scholars' researches, AEG-1 protein may be helpful for auxiliary diagnosis of bladder cancer and the judgment of patients' prognosis, which may become the new target for tumor gene therapy.

This study finds that Cyclin D1 protein in bladder cancer tissue cells are significantly higher than the tumor-adjacent tissue, suggesting that Cyclin D1 may participate in BUC tumorigenesis. Cyclin D1 protein expression in invasive carcinoma is much higher than superficial; The higher pathologic stage is, the higher the positive rate is, the more significant the positive rate of Cyclin D1 in the palindromic bladder cancer patients increased. These suggested

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that expression of Cyclin D1 is associated with the progression of bladder cancer. Many cancer patients have been found the overexpression of Cyclin D1 with poor prognosis [23, 24]. Our study also showed that patients with high Cyclin D1 expression showed significantly worse survival compared with those patients with low Cyclin D1 expression. It may be considered for auxiliary diagnosis of bladder cancer, and judging the prognosis of patients.

Wang find that AEG-1 has certain adjustment effect for Cyclin D1 protein [22]. This study shows that AEG-1 protein in bladder cancer up-regulated with higher expression of Cyclin D1, which meant that they are positively correlated. AEG-1 gene may through up-regulating the Cyclin D1 expression, leading to cell cycle disorder, and then promoting tumor cell proliferation.

Conclusion

In conclusion, protein of AEG-1 and Cyclin D1 are overexpression in BUC. And their overexpression correlates to poor prognosis in BUC patients. Detecting AEG-1 and Cyclin D1 may help for the auxiliary diagnosis of bladder cancer and judging the prognosis of patients. Further research about this is expected to be the new targets for gene therapy of BUC. However, this finding should be verified in a large sample size of BUC patients. Moreover, it needs to be further researched that AEG-1 and Cyclin D1 promote mechanisms of the tumorigenesis and progression in BUC.

Acknowledgements

This work is supported by (a) the 2015 basic and advanced technology research project of Henan Province, P. R. C. (No. 152300410177), and (b) Dr. Startup funds of Luohe Medical College, P. R. C. (No. 2013-DF-002).

Disclosure of conflict of interest

None.

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