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Original Article

AEG-1 acts as a novel bio-marker in the diagnosis of patients with hepatocellular carcinoma

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Abstract: Purpose: In order to find a novel non-invasive bio-marker with high accuracy for the diagnosis of hepatocellular carcinoma (HCC), we examined the predictive power of *AEG-1* as a potential bio-marker in HCC. Methods: A total of 184 serum samples (117 samples from HCC patients, 67 samples from healthy volunteers) were collected. We measured the levels of *AEG-1* with reverse transcription quantitative real-time polymerase chain reaction (qRT-PCR) in all specimens. χ^2 analysis was used to explore the correlation between the expression levels of *AEG-1* and clinicopathological parameters. In addition, receiver operating characteristic (ROC) curve analyses was performed to reveal whether the serum *AEG-1* could be promising diagnostic bio-marker for HCC. Results: The expression of *AEG-1* mRNA was significantly higher in HCC patients than healthy volunteers ($P<0.05$). *AEG-1* expression level was related with tumor size, tumor number, AJCC stage, T classification, N classification, M classification, histological differentiation, vascular invasion, distant metastasis and serum AFP levels ($P<0.05$), but had no relation with age and gender. ROC curve demonstrated that the optimal cutoff point was 0.733. Besides, the area under ROC curve (AUC) was 0.85, suggesting *AEG-1* could act as a diagnostic marker for HCC ($P=0.000$, 95% CI=0.791-0.908). Conclusion: Taken together, *AEG-1* mRNA was positively expressed in HCC serum compared to the healthy controls. *AEG-1* could be a novel serum bio-marker for HCC.

Keywords: Astrocyte elevated gene-1, hepatocelluar carcinoma, diagnosis

Introduction

Hepatocellular carcinoma (HCC) is not only the fifth most common cancer but also the third most common cause of cancer mortality worldwide [1, 2]. There are about 564,000 new HCC patients reported worldwide each year [3], 75-80% of HCC cases worldwide are from Asia [4], 55% of whom are Chinese [5, 6]. Due to the diagnosis at an advanced stage the rates of morbidity and mortality in HCC patients are equal [7-9]. Early diagnosis can be helpful to decrease deaths rates, but it is too difficult to conduct the early diagnosis of HCC because of the coexistence of inflammation and cirrhosis [10]. Therefore, it is crucial to explore a reliable clinical diagnostic method to improve the diagnostic efficiency [7].

Serum alpha-fetoprotein (AFP) level is a widely acknowledged bio-marker for HCC monitoring, but the sensitivity and specificity are not satisfactory [11]. In addition, some new specific markers have been identified, such as homeobox gene SMG-1 [12], GOLM1 [13], and Barx2 [14], in order to improve the sensitivity and specificity for early prediction of the prognosis of HCC. However, there is no confirmed evidence proving the diagnostic accuracy of these markers.

Therefore, identification of novel critical molecules that contribute to the progression of HCC would be extremely beneficial, not only for diagnostic/prognostic purposes but also for providing significant targets for therapeutic intervention. Astrocyte elevated gene-1 (*AEG-1*), a novel oncogene, also known as metadherin (MTDH) and lysine-rich CEACAM1 co-isolated (LYRIC) was first reported in 2002 as a novel late response gene following HIV-1 infection [15-18]. Within the last decade, overexpression of *AEG-1* has been detected in melanoma, breast and prostate cancers [19], ductal and lobular carcinomas [20], and malignant glioma cell lines compared with their normal counterparts

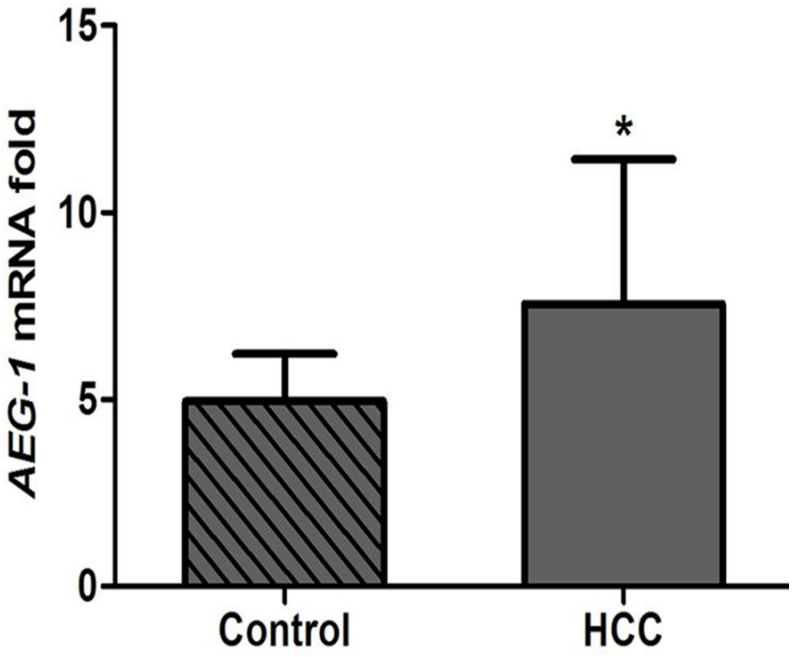

Figure 1. Relative expression levels of *AEG-1* in 67 controls and 117 HCC patients. The expression level of *AEG-1* in HCC tissues was higher than in the healthy specimens ($P<0.05$). The expression levels were determined using a qRT-PCR assay, and the relative expression data were analyzed using the $2^{-\Delta\Delta CT}$ method. All of the assays were performed in triplicate.

[19], and its elevated levels are associated with poor prognosis in cancer patients [21, 22]. *AEG-1* is over-expressed in 90% of human HCC patients and plays a significant role in mediating aggressive progression of HCC [23].

Previous studies have reported the involvement of abnormal expression of *AEG-1* in the prognosis of several other types of human cancers, such as gastric carcinoma [24], cervical cancer [25], tongue carcinoma [26] and osteosarcoma [27]. Excessive expression of *AEG-1* has been observed in several diseases, but little is reported about the expression of *AEG-1* related with diagnosis of HCC. The goals, in this present study, were to examine alternations of the expression of *AEG-1* in the serum of HCC patients and to evaluate whether the level of *AEG-1* could serve as a new bio-marker in the diagnosis of patients with HCC.

Materials and methods

Ethics statement

Ethical approval for this research was obtained from the Research Ethics Committee of Affiliated Hospital of Hebei University. All serum specimens and clinical materials were obtained and used with prior written informed consent from the patients and the Affiliated Hospital of Hebei University

Patients and tissue samples

We gathered a total of 184 serum samples, 117 out of which were from HCC patients, and the remaining samples were from healthy volunteers at Affiliated Hospital of Hebei University. All patients enrolled in this study were newly diagnosed and histopathologically confirmed by liver biopsy. 67 healthy volunteers matching ages, gender and residential area with HCC patients were diagnosed without any type of malignancy or other benign disease.

All of the blood specimens were obtained from those 184 persons, and those serum samples were taken on the day of diagnosis, prior to any surgeries and therapies. Then, the serum samples were stored at -80°C until analysis. Also the data of clinicopathological features of the HCC patients were collected including tumor size, tumor number, AJCC stage, T classification, N classification, M classification, histological differentiation, vascular invasion, serum AFP levels. Tumors were classified according to the 6th edition of the tumor-node-metastasis (TNM) classification of the International Union Against Cancer (UICC).

Serum preparation and RNA extraction

A volume of 5 ml of EDTA-anticoagulated blood was obtained from each patient and healthy volunteer. Serum was separated by centrifugation. Total RNA isolation was performed using a mirVana miRNA Isolation Kit (Ambion, Austin, Tex-as, USA) according to manufacturer's instructions. Extracted RNA samples were reverse transcription to cDNA as soon as possible, using an All-in-One First-Strand cDNA Synthesis Kit (Genecopoeia). Concentration and purification of RNA were carried out using

Table 1. Relationship between *AEG-1* expression and clinicopathological parameters in patients with HCC

	Parameters	No.	*AEG-1* expression level		*P* values
			Low	High	
Age (years)	≤55	84	24	60	0.853
	>55	33	10	23	
Gender	Male	87	25	62	0.895
	Female	30	9	21	
Tumor size (cm)	≤5	56	11	45	0.032
	>5	61	23	38	
Tumor number	≤2	56	24	32	0.002
	>2	61	10	51	
AJCC stage	I	60	24	36	0.026
	II	16	5	11	
	III	21	3	18	
	IV	20	2	18	
T classification	T1	47	20	27	0.037
	T2	24	7	17	
	T3	29	4	25	
	T4	17	3	14	
N classification	N1	63	24	39	0.02
	N0	54	10	44	
M classification	M1	73	27	46	0.015
	M0	44	7	37	
Histological differentiation	Good	71	27	44	0.029
	Moderate	32	5	27	
	Poor	14	2	12	
Vascular invasion	Absence	72	27	45	0.011
	Presence	45	7	38	
Distant metastasis	Yes	36	20	16	0.000
	No	81	14	67	
Serum AFP levels (μg/l)	≤400	65	29	36	0.000
	>400	52	5	47	

P<0.05 was considered as statistically significant (*P* values were calculated using the χ^2 test and the Fisher's exact test).

NanoDrop ND-1000 (NanoDrop, Wilmington, DE, USA). Only those total RNA samples with an OD A260/A280 ratio close to a value of 2.0, which indicated that the RNA was pure, were subsequently used.

Quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR)

The cDNA was synthesized using the Primescript RT Reagent kit (Takara, Dalian, China) using the oligo (dT) primer. RT-PCR reaction was performed in the Applied Biosystems 7900 Fast Real-Time PCR system (Applied Biosystems, Foster City, Ca-lifornia, USA). The expression of *AEG-1* at mRNA level was calculated via the comparative cycle threshold (CT) method. We evaluated all data by normalizing with the expression of GAPDH and using the $2^{-\Delta\Delta CT}$ method. The sequences of the primers were as follows: AEG-1, 5'-AAGAGGAAAACTGAGCCATCTG-3' (forward) and 5'-CGGCTAACATCCCACTGATAAT-3' (reverse); and GAP DH, 5'-GACTCATGACCACAGTCC-ATGC-3' (forwar) and 5'-AGAGGCAGGGATGAT-GTTCTG-3' (reverse). All of the specific primers used in the present study were designed and synthesized by Sangon Biotech (Shanghai, China). Each reaction was performed in triplicate. The specific reaction conditions were as follows: initial denaturation at 95°C for 1 min; template denaturation at 95°C for 20 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min (total of 40 cycles) and final extension at 72°C for 10 min.

Statistical analysis

All experiments were repeated at least three times. All statistical analyses were carried out using the SPSS statistical software program version 19.0 (SPSS Inc., Chicago, IL). All the data are summarized and presented as means ± standard deviation (SD). The differences between means were analyzed statistically using t-tests. The association between various clinicopathological factors and serum *AEG-1* expression were assessed using χ^2 analysis. The ROC curve analysis was performed to assess the diagnostic accuracy of *AEG-1*. The AUC was used to explore optimal sensitivity and specificity levels. When *P*<0.05, the difference was considered to be statistical significant.

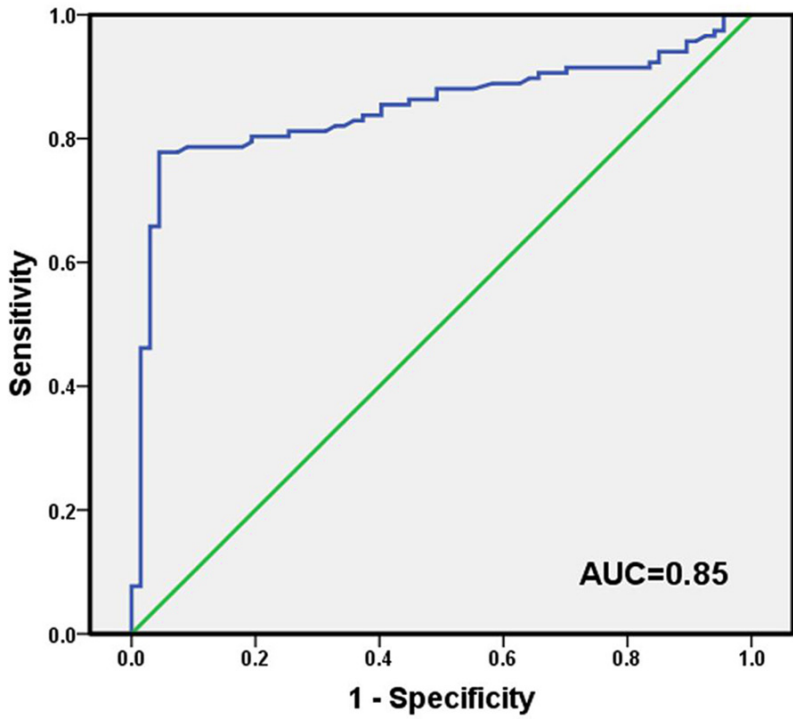

Figure 2. Receiver operating curve (ROC) analysis of *AEG-1* expression levels. The AUC was 0.941, suggesting that *AEG-1* was a diagnostic marker for HCC (*P*=0.000). ROC: receiver operating characteristic, AUC: area under the curve.

Results

Increased expression of AEG-1 in the serum of HCC

The mRNA expression level of *AEG-1* in 117 patients with HCC and 67 healthy volunteers were detected via qRT-PCR using GAPDH as normalization. As shown in **Figure 1**, the mRNA expression level of *TCF21* was significantly higher in HCC serum compared with healthy serum (*P*<0.05). The relative expression level of *AEG-1* mRNA in HCC patients was 7.555±0.357, while that of the controls was 4.959±0.154.

Relationship between AEG-1 and clinicopathological characteristics of HCC

In order to further investigate whether the expression level of *AEG-1* was correlated with the clini copathological feathers, we used Chi-square test to analyze. And all the data of clinicopathological feathers of the HCC patients were showed in **Table 1**. We found *AEG-1* expression was signi-ficantly associated with tumor size (*P*=0.032), tumor number (*P*=0.002), AJCC stage (*P*=0.026), T classification (*P*=0.037), N classification (*P*=0.02), M classification (*P*=0.015), histological differentiation (*P*=0.029), vascular invasion (*P*=0.011), distant metastasis (*P*=0.000) and serum AFP levels (*P*=000).However, there was no association between HCC expression and patients' age and gender (all *P*>0.05).

Diagnostic value of serum AEG-1 for HCC

ROC cures were used to verify the diagnostic accuracy of serum *AEG-1* for HCC. The optimal cutoff point was determined according to the maximum sum of specificity and sensitivity. As shown in **Figure 2**, the optimal cutoff point was 0.733, providing the specificity and sensitivity of 100% and 82.9%, respectively. Besides, the area under ROC curve (AUC) was 0.85. All the statistical data displayed that *AEG-1* has great diagnostic value for HCC (*P*=0.000, 95% CI=0.791-0.908).

Discussion

HCC is a complex, multifactorial disease caused by a variety of factors, such as hepatitis B virus infection, alcohol consumption, cigarette smoking, obesity, and chronic viral hepatitis [28, 29]. The most striking feature of HCC is rapid growth, early vascular invasion, high-grade malignant potential and multidrug resistance, so early diagnosis and treatment are essential for HCC management [1, 30, 31]. At present, the pathologic confirmation is the gold standard for HCC diagnosis. Besides, the widely applied method is testing the serum AFP level combined with imaging techniques, including ultrasonography, magnetic resonance imaging, and computerized tomography [32, 33]. However, the sensitivity and specificity of serum AFP level are relatively unsatisfactory.

AEG-1 is a single-pass transmembrane protein [19]. Recent studies have confirmed that AGE-1 inhibited cancer cell apoptosis and increases invasiveness and metastasis [34-36]. It regu-

lates different signaling pathways that are closely related to cancer, such as nuclear factor-kappa B, Wnt/β-catenin, MAPK/ERK, PI3K/Akt, and AP-1 [23, 36-38]. Clinical studies have linked *AEG-1* with tumor progression and poor clinical outcomes in several cancer types, including breast cancer, prostate cancer, esophageal cancer, colorectal carcinoma, and HCC [38-42]. Besides, some research reported that *AEG-1* is lowly expressed in normal tissues, with higher expression detected in HCC [43], breast [44], gastric [45], gallbladder [46], colorectal [47], prostate [48] and renal [49] cancer. Increasing evidence has demonstrated that *AEG-1* is involved in cancer diagnosis.

In the present study, clinicopathological survey showed that the serum *AEG-1* level was associated with tumor size, tumor number, AJCC stage, T classification, N classification, M classification, histological differentiation, vascular invasion, distant metastasis and serum AFP levels. After the ROC curve analysis, the results revealed that *AEG-1* may be a potential serum biomarker to distinguish HCC patients from non-cancer patients.

In conclusion, these findings provide the convincing evidence for the first time that the upregulation of *AEG-1* might serve as a novel molecular marker for the diagnosis of HCC. However, there are still some limitations. Firstly, the sample size is small. To solve this problem, further validating and improving study with larger sample should be conducted to confirm our results. Secondly, the current study has not elucidated the exact molecular mechanisms of *AEG-1* acting on HCC, which is also worth to be further investigated.

Disclosure of conflict of interest

None.

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