Upregulation of AUF1 is involved in the proliferation of esophageal squamous cell carcinoma through GCH1

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Abstract. Esophageal squamous cell carcinoma (ESCC) has one of the highest mortality rates worldwide. AU-rich element RNA-binding factor 1 (AUF1) is an established RNA-binding protein. AUF1 influences the process of development, apoptosis and tumorigenesis via interacting with adenylate-uridylate rich elements (AREs) bearing mRNAs. However, the clinical relevance of AUF1 and its biological function in ESCC progression have not been reported. In the present study, we first investigated the expression of AUF1 in the ESCC tissue samles and normal samples. We found a significantly higher expression of AUF1 in ESCC tissues than that in normal tissues and tumor adjacent tissues. The expression of AUF1 correlated with ESCC stage (P=0.011) and marginally correlated with lymph node metastasis (P=0.055) of ESCC patients. Silencing of AUF1 by an siRNA inhibited the proliferation and enhanced the apoptosis of ESCC cells. mRNA profiling by microarray analysis revealed that AUF1 knockdown affected 285 genes (fold change ≥2) that function in multiple pathways. GTP cyclohydrolase I (GCH1), the rate limiting enzyme for BH4 synthesis, was found to be down-regulated. One of the AU-rich elements in the 3'UTR of GCH1 was found to be responsive to AUF1 expression by luciferase assay. Knockdown of GCH1 suppressed cell proliferation and colony formation of ESCC cells. The expression of AUF1 significantly correlated with that of GCH1 in ESCC tissues. Taken together, we demonstrated the overexpression of AUF1 in esophageal carcinoma and identified GCH1 as AUF1's effector for the proliferation of ESCC cells.

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Introduction

Esophageal cancer is the sixth leading cause of cancer death in male and the ninth in female, with an estimated over 400,000 new cases and deaths each year worldwide (1). The two main histological types are esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma. ESCC is the most common histological subtype in China with 95% of all esophageal cancer cases. With new methods of diagnosis and treatment, esophageal cancer still has a poor 5-year survival rate (2). Most of their mortality is not due to their primary tumors, but to their invasiveness and metastasis (3). A better understanding of molecular pathogenesis of ESCC is urgent for the diagnosis and treatment of the disease.

Regulation of gene expression is a major control point in organisms to adapt to the environment. The regulation of mRNA stability and translation are the basis of eukaryotes in response to intrinsic or extrinsic stimuli. Many mRNAs encode proto-oncogenes and cytokines, the aberrant mRNA stability is associated with inflammation and carcinogenesis (4,5). It is recognized that approximately 16% of all human protein coding genes are encoded by mRNAs that contain an adenylate-uridylate (AU)-rich element (ARE) motif in their 3'UTR (6-9). AU-rich element RNA-binding factor 1 (AUF1), also called the heterogeneous nuclear ribonucleoprotein D (hnRNPD), is an established RNA-binding protein. The

human AUF1 locus maps to 4q21, and it comprises a family of proteins composed of four isoforms with different molecular masses: p37, p40, p42 and p45 (10). AUF1 isoforms express preferentially in different tissues, which have their distinct biological properties (10). AUF1 influences the process of development, apoptosis and tumorigenesis via interacting with ARE-bearing mRNAs (11).

Altered AUF1 expression level may be a promoter to the development and progression of multiple types of cancers. It was reported that overexpression of AUF1 remarkably affected the stability of ARE containing mRNAs. Kumar *et al* (12) found that the expression level of nuclear AUF1 was significantly higher in oral squamous cell carcinoma than that in normal cells, and downregulated AUF1 with RNAi resulted in decreased proliferation rates. Overexpression of AUF1 isoform p37 developed sarcomas with deregulated c-Myc, c-Fos and c-Jun and cyclin D1 expression in tumor tissues (13). AUF1 has been shown to facilitate the decay of multiple target mRNAs, but occasionally to modulate the stability and translation of several target mRNAs (14). Several studies have shown that AUF1 plays an anti-tumorigenic role in regulation of the cell cycle and apoptosis. For example, increasing the AUF1 by UVC may promote apoptosis by reducing ARE-containing Bcl-2 mRNA (15). Treatment with postaglandin A2 (PGA2), an anticancer agent, induces expression of AUF1 isoform p45 and leads to the destabilization of cyclin D1 mRNA in non-small cell lung cancer cells (16). The above evidence suggests that AUF1 may be a promoter or inhibitor in the processes of carcinogenesis or proliferation, which vary between different types of cancer cells.

The clinical relevance of AUF1 and its biological function in ESCC progression have not been reported. In the present study, we investigated the expression of AUF1 in the ESCC tissue samles and normal samples. We further illustrated the role and downstream effector of AUF1 in driving cell proliferation of ESCC cells.

Materials and methods

Tissue samples. The ESCC samples were collected from 104 patients who had not received chemotherapy or radiotherapy prior to surgery. For these patients, if preoperative ultrasonography or computed tomography (CT) did not show any enlarged cervical lymph nodes (minor axis <0.5 cm), patients underwent a subtotal esophagectomy with two-field lymphadenectomy through a right thoracotomy, followed by a laparotomy. If enlarged cervical lymph nodes were detected by preoperative ultrasonography or CT, patients underwent radical oesophagectomy with cervico-thoraco-abdominal three-field lymphadenectomy through a right thoracotomy, followed by a laparotomy and a cervical incision. The resection extent includes nodes along the cervical part of the esophagus and deep cervix. Tumor adjacent tissues were obtained 3 cm away from the tumor. Fifteen normal esophageal tissue samples were obtained from surgical resections of trauma patients. These normal esophageal tissue samples were obtained from upper endoscopy of trauma patients, which were performed by the same operator. These patients had a fish bone or a poultry bone stuck. These tissues were obtained postoperatively between 2010 and 2013 from the Gastrointestinal Center, Jiangyin People's Hospital, the Jiangyin Clinical College of Xuzhou Medical University (Jiangyin, China) as previously reported (17). All patients provided signed and informed consents for their tissue samples to be used for scientific research. The ethics approval for the study was obtained from the Jiangyin People's Hospital, the Jiangyin Clinical College of Xuzhou Medical University. All diagnoses were based on pathological and/or cytological evidence. The histological features of the specimens were evaluated by a senior pathologist according to the classification criteria from the World Health Organization (18). Tissue samples were obtained prior to chemotherapy and radiation therapy and were immediately fixed in 10% neutral buffered formalin prior to immunohistochemistry analysis.

Immunohistochemistry staining (IHC). The tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin. Three-micrometer thick paraffin sections were deparaffinized and heat-treated with citrate buffer (pH 6.0) for 7 min as an epitope retrieval protocol. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 15 min at room temperature and tissue non-specific binding sites were blocked with skim milk powder at 4% applied for 30 min. Sections were then incubated with the AUF1 and GCH1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h and mixed with skim milk powder at 2% again to reduce the non-specific staining. Biotinylated secondary antibody was then added for 30 min. IHC staining was visualized with substrate solution containing diaminobenzidine (DAB) and hydrogen peroxide. The counter-staining was performed with haematoxylin. All steps were performed at room temperature. Negative controls consisted of tissue sections undergoing similar staining procedures in the absence of the primary antibody. The criteria for scoring the stained sections were as follows: negative (0), <10% of the whole tissue section stained positive; weakly positive (+ 1), 10-25% of the whole tissue section stained positive; moderately positive (+ 2), 25-75% of the whole tissue section stained positive; and strongly positive (+ 3), 75% of the tissue section stained positive. Tissues with score 0 or 1 were considered as AUF1-negative, while with score 2 or 3 were considered as AUF1-positive.

Cell culture and transfection. The human esophageal cancer cell line Eca-109 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (Gibco, Grand Island, NY, USA). Cells were grown in a 37˚C incubator with 5% CO_2.

The siRNA targeting AUF1 (siRNA-AUF1) and siRNA control (siRNA-NC) used in the present study were obtained from Santa Cruz Biotechnology. The cells were grown in a 6-well culture plate to 70-80% confluence and then transfected with either siRNA-NC, siRNA-AUF1 or siRNA-GCH1 (Santa Cruz Biotechnology) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 48 h, the cells were collected for western blot analysis.

Cell viability assay. Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Cells were seeded in 96-well plates.

Table I. Primer sequences for real-time PCR analysis.

Gene	Forward primer	Reverse primer
GAPDH	5'-AAGGTGAAGGTCGGAGTC-3'	5'-GAAGATGGTGATGGGATTTC-3'
Nalcn	5'-CCATGAAATCTGTCGTGTGC-3'	5'-TGCTCACTGGACAATCAAGG-3'
UGP2	5'-TCTGGAAACCTTCGCATCTT-3'	5'-TTGCTCCATCTGTGCTTTTC-3'
BEX2	5'-TAATAAAGGGGAGCCCTTGG-3'	5'-ACTGCCCGCAAACTATGACT-3'
Prdm16	5'-ACGCTTGGTTGAAGGTCACT-3'	5'-TGGAAAAGTCGGAATGAACC-3'

The next day, the cells were transfected with plasmids or siRNAs according to the experimental design. The cells were then incubated with 20 μl MTT (5 mg/ml) for 4 h. After the medium was removed, 100 μl DMSO was added and the optical density (OD) at 490 nm was measured using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The viability index was calculated as experimental OD value/control OD value. Three independent experiments were performed in quadruplicate.

Focus formation. Cells transfected with the indicated vectors were plated at low density (1,000 cells/6-cm plate), incubated for 10 days and fixed and stained with crystal violet. Foci and colonies containing >50 cells were counted using a microscope.

Western blot analysis. After transfection, cells were harvested and lysed in RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 4˚C. Protein (40 μg) from each lysate was fractionated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocking with 5% non-fat milk in PBS-Tween-20 for 1 h at room temperature, the membranes were blotted with AUF1 and GCH1 primary antibodies (Abcam, Cambridge, UK). GAPDH (Beyotime Institute of Biotechnology, Nantong, China) was used as a loading control. After washing four times with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h. The proteins were visualized using the enhanced chemiluminescence (ECL; Beyotime Institute of Biotechnology).

Microarray analysis. Microarray-based mRNA expression profiling was performed using the Roche-NimbleGen (135K array) array (Roche NimbleGen, Madison, WI, USA). The microarrays contained approximately 45,033 assay probes corresponding to all of the annotated human mRNA sequences (NCBI HG18). Total RNA labeling and hybridization were performed using standard conditions according to the manufacturer's instructions. Genes with fold change of 2 or greater were subsequently subjected to pathway analysis using Ingenuity Pathway Analysis (Qiagen, Redwood City, CA, USA).

RNA extraction and real-time PCR. The total RNA from the esophageal tissue samples was extracted with TRIzol (Invitrogen) and reverse transcribed to complementary DNA (cDNA) using an oligo$(dT)_{12}$ primer and SuperScript II (Invitrogen). The SYBR-Green dye (Takara Bio, Osaka, Japan) was used for the amplification of cDNA. The mRNA levels of transcripts were measured using real-time quantitative PCR in triplicate on a Prism 7500 real-time PCR machine (Applied Biosystems, Foster City, CA, USA). Primers specific for the genes are listed in Table I. PCR was performed in a 20 μl reaction mixture. The thermal cycling conditions included an initial denaturation step of 95˚C for 20 sec, followed by 40 cycles at 95˚C for 5 sec and annealing at 60˚C for 30 sec. The relative mRNA expression levels were normalized to *GAPDH*.

Luciferase assays. Two luciferase reporters containing the full-length 3'UTR fragment of the *GCH1* 3'UTR (+1 to +1063, relative to the stop coden) was constructed by Shanghai Biobuy Co., Ltd. (Shanghai, China). Then, constructs each with a deletion of the two potential AU-rich elements in the *GCH1* 3'UTR were constructed. For the luciferase assay, the reporter vector with the *GCH1* 3'UTR was co-transfected with pRL-TK (Promega, Madison, WI, USA) to correct the transfection efficiency. Luciferase activity was measured with the Dual-luciferase reporter assay system (Promega). Promoter activities were expressed as the ratio of firefly luciferase to *Renilla* luciferase activities.

Intracellular nitric oxide (NO) determination. Total NO concentration in cultured cells was detected by measuring the concentration of nitrate and nitrite by modified Griess reaction method. After lysis of cells, a total Nitric Oxide assay kit (Beyotime Institute of Biotechnology) was used to detect NO. The optical densities at 540 nm wavelength were recorded using a microplate reader (BioTek Instruments, Winooski, VT, USA) and the concentrations of NO were calculated according to the standard curve.

Statistical analysis. Data are expressed as the means ± standard error of the mean (SEM) of at least three independent experiments. Standard error bars were included for all data points. The data were first analyzed with the Kolmogorov-Smirnov test for data distribution normality. The data were then analyzed using Student's t-test when only two groups were present or assessed by one-way analysis of variance (ANOVA) when more than two groups were compared. The P-values for t-tests were performed by 2-tailed t-tests. Correlation analysis of the mRNA expression data was performed using the Pearson test. Statistical analysis was

Table II. Patient demographic features for the IHC analysis.

	Normal	Grade I	Grade II	Grade III	Grade IV	All tumors
Number	15	15	52	33	4	104
Mean age (years)	58.73	62.88	59.21	56.50	66.00	58.90
Age range (years)	32-65	55-69	33-73	32-75	52-67	32-76
Gender						
Male	10	9	37	23	4	73
Female	5	6	15	10	0	31

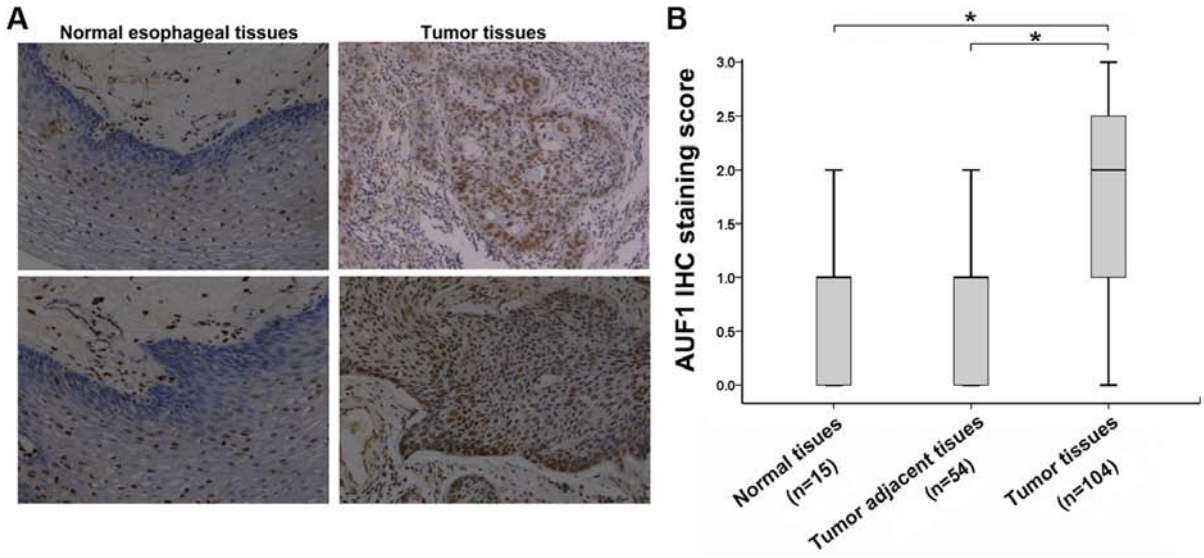

Figure 1. Upregulation of AUF1 in ESCC samples. (A) Representative IHC staining of AUF1 expression in normal esophageal tissue specimen and ESCC tissues (magnification, x100). (B) Box plot representing the range of AUF1 immunohistochemical (IHC) staining score in normal esophageal tissues (n=15), ESCC tissues (n=104) and tumor adjacent tissues (n=52). *P<0.05.

performed using SPSS software (Release 19.0; SPSS, Inc., Chicago, IL, USA). Data were considered significant at P<0.05.

Results

Upregulation of AUF1 is correlated with clinicopathological feature in esophageal squamous cell carcinoma. To investigate the expression of AUF1 in human ESCC tissues, we performed immunohistochemistry analysis on tissue section of normal esophageal tissue specimens, ESCC and tumor adjacent tissues. Fifteen paraffin-embedded normal esophageal tissues and 104 ESCC tissues, 54 of which had corresponding adjacent tissues were collected. The demographic features of patients with ESCC for the immunohistochemistry staining are summarized in Table II. Results from IHC staining showed that a generally stronger staining of AUF1 was observed in the nucleus of ESCC tissues, whereas very weak staining of AUF1 was observed in the normal samples (Fig. 1A). When the samples were scored by a pathologist, as described in Materials and methods, AUF1 expression was similar between normal esophageal tissues and tumor adjacent tissues, while increased significantly among ESCC tissues, and between tumor tissues and tumor adjacent tissues (P<0.05; Fig. 1B). All the above results indicate that AUF1 may be involved in the pathogenesis of ESCC.

Next, the relationship between clinicopathology of ESCC patients and AUF1 IHC staining score was analyzed. As shown in Table III, the results showed a non-significant correlation between AUF1 expression and age (P=0.610), gender (P=0.637) and lymphatic invasion (P=0.096) of ESCC patients. AUF1 expression correlated with ESCC stage (P=0.011) and marginally correlated with lymph node metastasis (P=0.055). These results suggest AUF1 as a characteristic of this malignancy.

AUF1 expression affects the proliferation and apoptosis of ESCC cells. Because AUF1 expression was associated with ESCC progression, we sought to explore the role of AUF1 in esophageal cancer cell growth. Eca-109 cells were transfected with a siRNA control (siRNA-NC) or siRNA trageting AUF1 (siRNA-AUF1). As shown in Fig. 2A, the protein level of AUF1 was markedly reduced after transfection of siRNA-AUF1. Next, the effect of AUF1 on cell growth was measured with MTT and colony formation. The results showed that decreased AUF1 expression suppressed cell growth and focus formation (Fig. 2B and C). We next investigated whether reduced cell growth was associated with apoptosis in ESCC cells. As

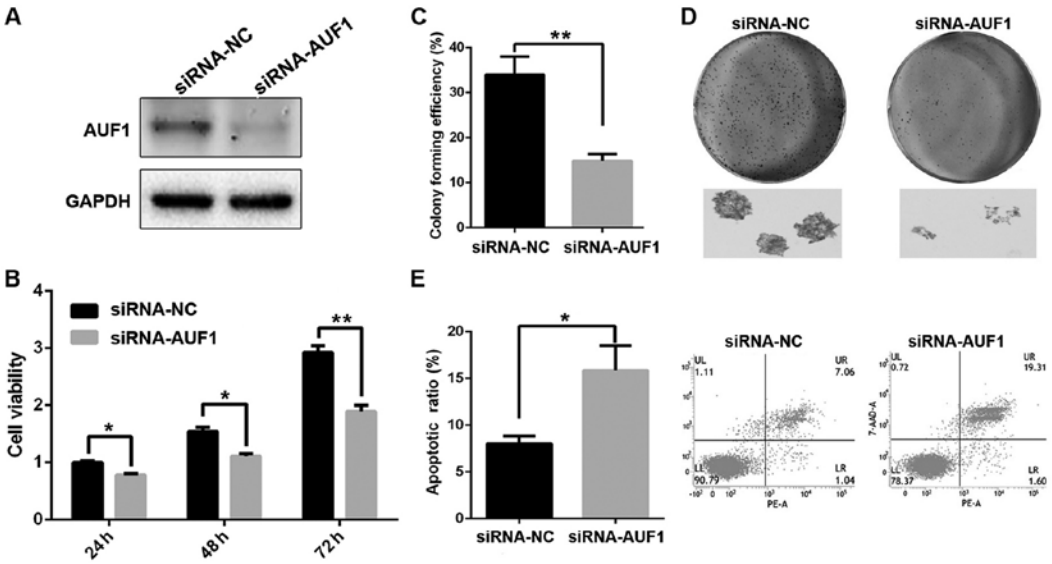

Figure 2. The effect of AUF1 on cell proliferation and focus formation. (A) Eca-109 cells were transfected with siRNA-AUF1 or siRNA-NC. The protein level of AUF1 was measured by western blot analysis. (B) Eca-109 cell lines were seeded in a 96-well plate and incubated for 48 h. Cells were transfected with siRNA-control (siRNA-NC) or siRNA-AUF1. Cell proliferation was measured by MTT. (C and D) The effect of AUF1 on Eca-109 cell focus formation. Eca-109 cells were transfected with siRNA-AUF1 or siRNA-NC. One thousand cells were seeded onto each plate. After 10 days, cells were stained with crystal violet. Colonies consisting of >50 cells were counted. The upper panel shows the representative focus, and the lower panel shows the representative clones. (E) Cell apoptosis rate was measured using Annexin V/7-AAD double staining. Data are presented as means ± SEM and were normalized to the control cells. *P<0.05 and **P<0.01, compared with the control group.

Table III. Relationship between clinicopathological parameters and the expression of AUF1 in esophageal squamous cell carcinoma (ESCC) (n=104).

Clinicopathological parameters	Case	AUF1 expression		P-value
		Negative	Positive	
Age (years)				0.610
<64	61	22	39	
≥64	43	13	30	
Gender				0.637
Male	73	25	48	
Female	31	10	21	
Stage				0.011
1	15	9	6	
2	52	18	34	
3	33	7	26	
4	4	1	3	
pN				0.055
(+)	41	9	32	
(-)	63	26	37	
Lymphatic invasion				0.096
(+)	49	12	37	
(-)	55	23	32	

pN, lymph node metastasis.

shown in Fig. 2E, silencing of AUF1 significantly increased apoptosis of Eca-109 cells. These results demonstrated that AUF1 modulates the proliferation and apoptotic cell death of ESCC cells.

mRNA microarray screening of AUF1-affected genes. Although many AUF1-regulated genes have been identified, the profile of AUF1-affected genes in ESCC cells remains elusive. To further analyze the underlying mechanisms responsible for AUF1-mediated effects, we screened gene expression between AUF1 silencing (siRNA-AUF1) and the control cells (siRNA-NC) by microarray. Twenty-four hours after the transfection, a total of 285 genes (138 upregulated and 147 downregulated genes) were identified with an expression differential of 2-fold or greater between the two groups (n=2; Fig. 3A and Table IV). The differentially expressed genes included Nalcn, MMP23B, E2F1, BEX2 and GCH1. We then validated the results from microarray analysis by RT-PCR. As shown in Fig. 3B, compared with control cells, several genes dysregulated in microarray data, such as Nalcn, UGP2, BEX2 and Prdm16 showed consistent changes by real-time PCR analysis. As expected, AUF1 appeared to have modulated the Eca-109 cells via complex mechanisms. Pathway analysis revealed that AUF1 affected multiple pathways, including MAPK signaling pathway, transcriptional regulation and adipocytokine signaling pathway (Fig. 3C). These results suggested that AUF1 might be a wide-spectrum regulator of gene expression in esophageal cancer cells.

AUF1 regulated GCH1 expression through 3'UTR. Among AUF1 affected genes, we focused on GCH1, which catalyzes the formation of 5,6,7,8-tetrahydrobiopterin (BH4), an essential co-factor for all nitric oxide synthases (19,20). GCH1 has been reported to contribute to cancer progression (21). Sequence analysis of the GCH1 3'UTR identified two potential AU-rich

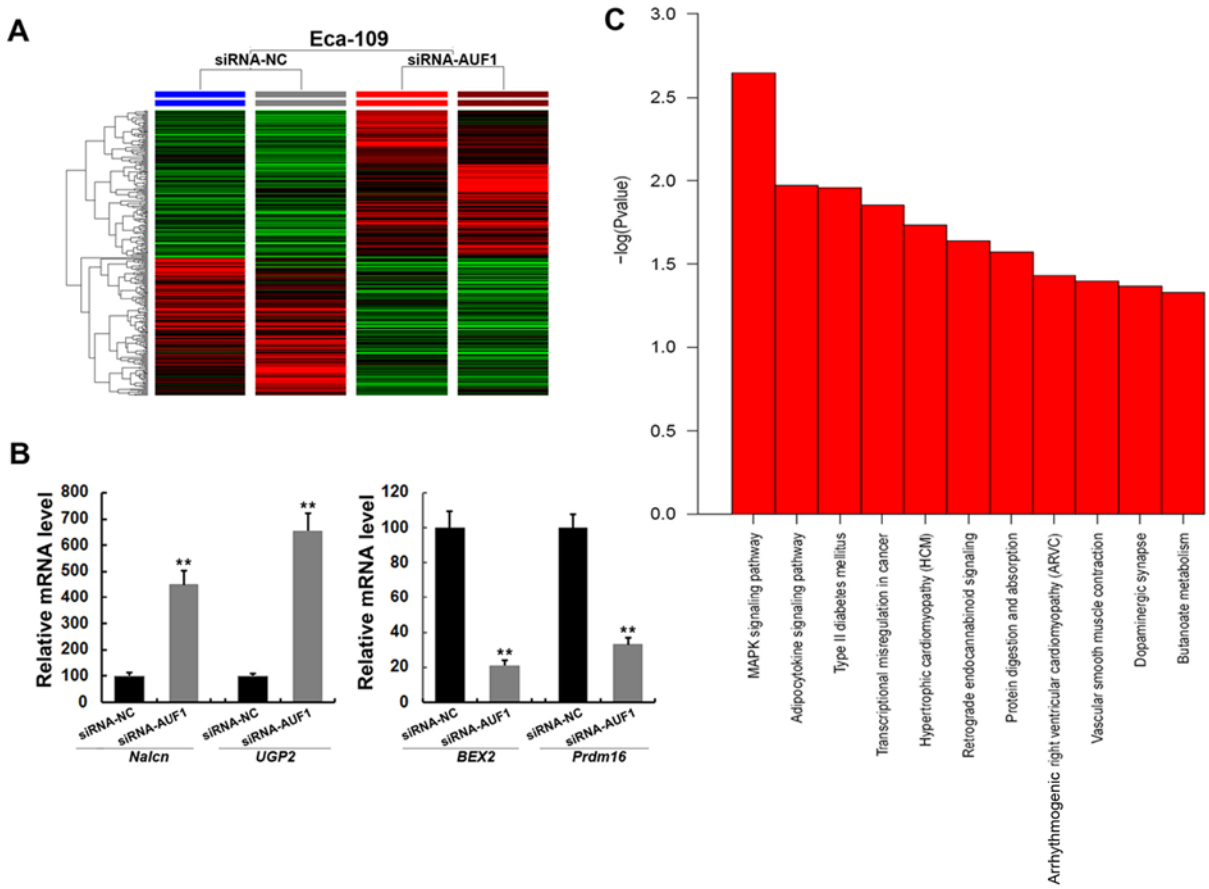

Figure 3. Predicted significant pathways affected by AUF1 overexpression. Cells were transfected with siRNA-AUF1 or siRNA-NC. RNA samples were collected and subjected to mRNA micorarray analysis. (A) Heatmap of gene expression between siRNA-NC- and siRNA-AUF1-transfected Eca-109 cells. (B) Real-time PCR analysis of *Nalcn*, *UGP2*, *BEX2* and *Prdm 16* in the control and AUF1 silenced Eca-109 cells. (C) Predicted significant pathways for dysregulated genes.

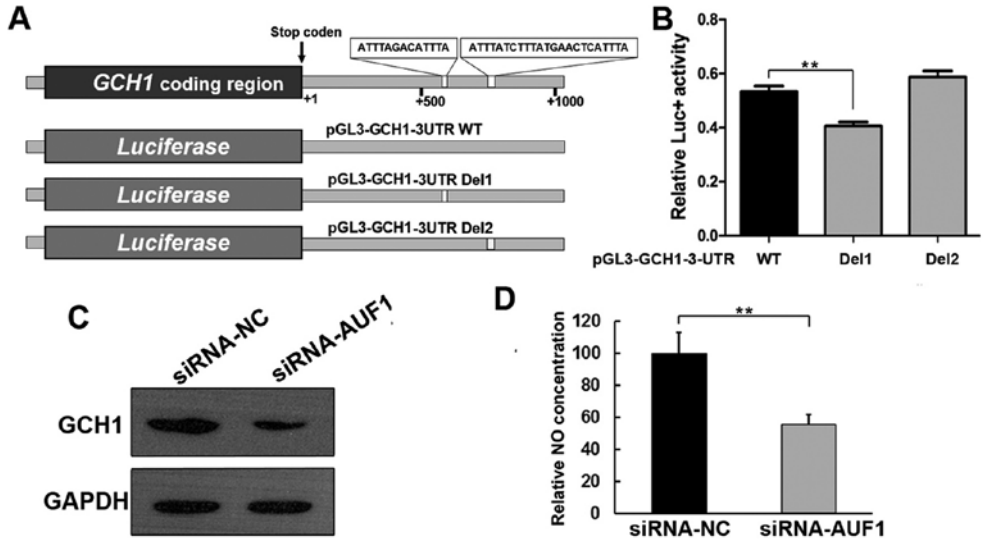

Figure 4. AUF1 positively regulates GCH1 in esophageal cancer cells. (A) The diagram indicates the relative positions of potential AU-rich elements (ARE) in the *GCH1* 3'UTR. Wild-type *GCH1* 3'UTR or deletion of each ARE in *GCH1* 3'UTR were cloned downstream of the firefly luciferase reporter gene. (B) Eca-109 cells were transfected with equivalent indicated reporters. After 24 h of incubation, luciferase activity was measured using the Dual-luciferase reporter assay system (Promega). (C) Eca-109 cells were transfected with siRNA-NC or siRNA-AUF1. Western blot analysis of GCH1 expression after silencing of AUF1. (D) The NO concentration was measured using the Griess assay. $^{**}P<0.01$ compared with the control group.

elements (Fig. 4A), which were likely to be regulated by AUF1. We therefore investigated whether AUF1 modulated the expression of GCH1 and cancer progression through GCH1. The effect of AUF1 on the *GCH1* 3'UTR was also measured

Table IV. Micorarray analysis of gene expression changes between siRNA-NC- and siRNA-AUF1-transfected cells.

Gene name	Fold change upregulated	Chromosome	Description
NALCN	25.949	chr13	Sodium leak channel, non selective
UGP2	20.386	chr2	UDP-glucose pyrophosphorylase 2
SCAF1	17.645	chr19	SR-related CTD-associated factor 1
KRTAP10-10	17.180	chr21	Keratin associated protein 10-10
MMP23B	16.273	chr1	Matrix metallopeptidase 23B
MAP7D2	15.894	chrX	MAP7 domain containing 2
TMEM129	15.310	chr4	Transmembrane protein 129, E3 ubiquitin protein ligase
DACT1	15.130	chr14	Dishevelled-binding antagonist of β-catenin 1
ARHGAP30	13.292	chr1	Rho GTPase activating protein 30
E2F1	13.152	chr20	E2F transcription factor 1
PCNXL2	12.669	chr1	Pecanex-like 2 (*Drosophila*)
NID1	12.587	chr1	Nidogen 1
PAK6	11.838	chr15	P21 protein (Cdc42/Rac)-activated kinase 6
AMBRA1	11.740	chr11	Autophagy/Beclin-1 regulator 1
TAF11	11.715	chr6	TAF11 RNA polymerase II, TATA Box binding protein (TBP)-associated factor, 28 kDa
NCDN	11.146	chr1	Neurochondrin
C14ORF101	11.047	chr14	Transmembrane protein 260
WDR20	10.609	chr14	WD repeat domain 20
CCDC74A	10.475	chr2	Coiled-coil domain containing 74A
TRO	10.257	chrX	Trophinin
Gene name	**Fold change downregulated**	**Chromosome**	**Description**
BEX2	23.010	chrX	Brain expressed X-linked 2
PRDM16	19.479	chr1	PR domain containing 16
ARX	18.122	chrX	Aristaless related homeobox
CPZ	17.834	chr4	Carboxypeptidase Z
GRM4	17.232	chr6	Glutamate receptor, metabotropic 4
PHF20L1	16.919	chr8	PHD finger protein 20-like 1
ANO9	13.588	chr11	Anoctamin 9
SLC24A4	13.212	chr14	Solute carrier family 24
BAZ1B	12.677	chr7	Bromodomain adjacent to zinc finger domain, 1B
C1ORF147	11.959	chr1	Chromosome 1 open reading frame 147
SIRT2	10.788	chr19	Sirtuin 2
RCBTB1	10.731	chr13	Regulator of chromosome condensation (RCC1) and BTB (POZ) domain containing protein 1
ISM1	10.317	chr20	Isthmin 1, angiogenesis inhibitor
OAZ2	10.129	chr15	Ornithine decarboxylase antizyme 2
SNCAIP	10.088	chr5	Synuclein, α interacting protein
CAMK1G	10.031	chr1	Calcium/calmodulin-dependent protein kinase IG
MYCT1	9.578	chr6	Myc target 1
SEC14L3	8.865	chr22	SEC14-like lipid binding 3
SGCB	8.732	chr4	Sarcoglycan, β
GCH1	8.726	chr14	GTP cyclohydrolase 1

using luciferase constructs containing the *GCH1* 3'UTR with or without AUF1 binding sites (Fig. 4A). The results revealed that deletion of the ARE1 (pGL3-GCH1 3UTR Del1) showed significantly decreased luciferase activity compared with that of wild-type GCH1 3'UTR in Eca-109 cells (Fig. 4B). However, deletion of the ARE2 (pGL3-GCH1 3UTR Del2) showed similar luciferase activity as compared with that of wild-type GCH1 3'UTR in Eca-109 cells (Fig. 4B). Western

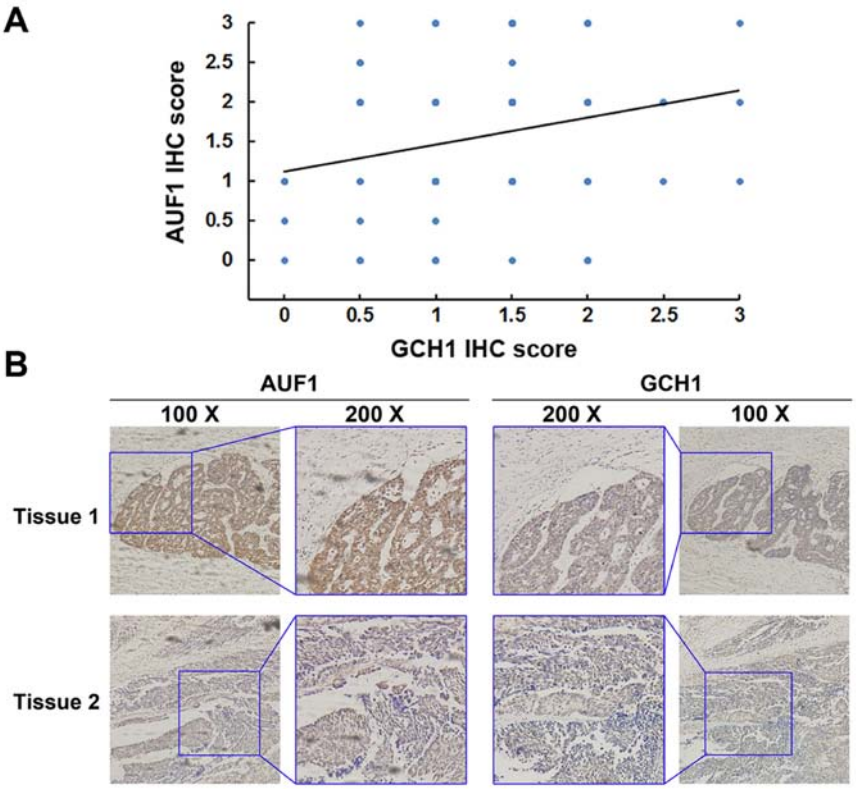

Figure 5. Correlation analyses between AUF1 and GCH1 expression in tissue samples. Immunohistochemistry (IHC) was used to detect the expression levels of AUF1 and GCH1 tissue samples. (A) Correlation between AUF1 and GCH1 in 104 ESCC tissue samples. AUF1 positively correlated with GCH1 in esophageal tissue samples. (B) Representative IHC staining of both AUF1 and GCH1 in the same tissue.

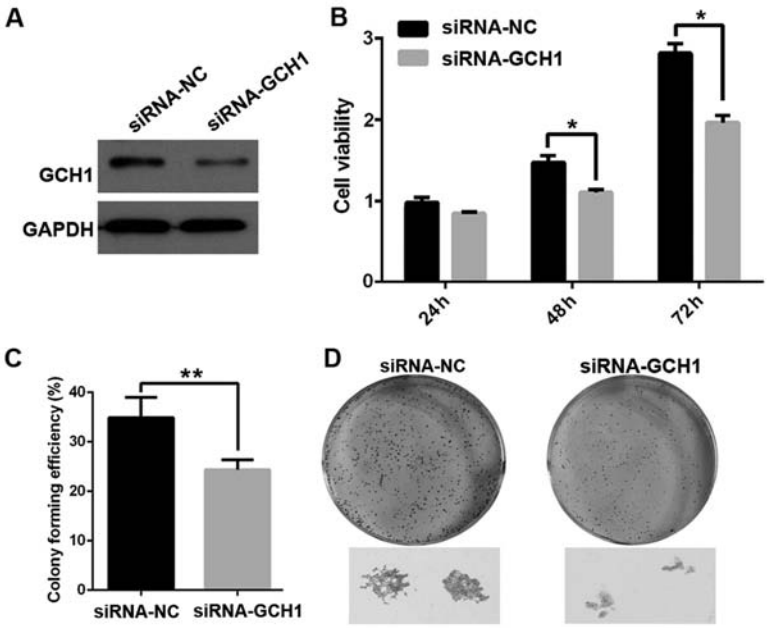

Figure 6. The effect of GCH1 on cell proliferation and focus formation of ESCC cells. Eca-109 cells were seeded in a 96-well plate and incubated for 48 h. (A) Cells were transfected with siRNA-control (siRNA-NC) or siRNA-GCH1. (B) After the transfection, cell proliferation was measured by MTT. Data are presented as means ± SEM and were normalized to the control cells, $^{*}P<0.05$; $^{**}P<0.01$. (C and D) The effect of AUF1 on Eca-109 cell focus formation. Eca-109 cells were transfected with siRNA-control (siRNA-NC) or siRNA-GCH1. One thousand cells were seeded onto each plate. After 10 days, cells were stained with crystal violet. Colonies consisting of more than 50 cells were counted. The upper panel shows the representative focus, and the lower panel shows the representative clones.

blot analysis further confirmed that knockdown of AUF1 decreased the expression of GCH1 (Fig. 4C). Because GCH1 is an essential co-factor for all NOS, as expected, silencing of AUF1 significantly reduced cellular NO level. These results

suggested that AUF1 regulated GCH1 through ARE1 region (Fig. 4D).

Expression of AUF1 correlates with GCH1 in ESCC tissues. The above mentioned results led us to investigate whether the expression level of AUF1 correlated with that of GCH1 in tissue samples. Immunohistochemistry was used to detect the expression levels of GCH1 protein in 102 ESCC tissue samples. Representative IHC staining of both AUF1 and GCH1 in the same tissue is shown in Fig. 5A. Tissue samples with strong AUF1 staining in the nucleus exhibited positive GCH1 staining, whereas weak AUF1 expression showed negative GCH1 staining. Statistical analysis revealed that the protein levels of the two genes correlated with each other (Pearson r=0.251, P=0.019; Fig. 5B), indicating that the expression of AUF1 correlates with the expression of GCH1 in human ESCC tissues.

Inhibition of GCH1 suppresses the proliferation and colony formation in ESCC cells. To explore the role of GCH1 in esophageal cancer cell growth, cells were first transfected with a siRNA-NC or siRNA-GCH1, and the resulting growth was measured with MTT and colony formation assay. The results showed that GCH1 silencing caused significantly decreased cell viability (75% of the control group). Moreover, knockdown of GCH1 attenuated the number and the size of colonies of Eca-109 cells. These results suggest that GCH1 promotes esophageal tumor growth *in vitro*. (Fig. 6).

Discussion

AUF1 plays a central role in various physiological and pathological processes. Excessive and dysregulated AUF1 expression has been implicated in diseases including cancers. Overexpression of AUF1 has been detected in numerous malignancies, including breast, skin, thyroid, liver, oral squamous cell carcinoma and sarcoma (12,13,16,22-24). AUF1 has therefore been recognized to modulate different cancer-related events. However, there is considerable controversy in defining its role in cancer progression. Several studies have shown that AUF1 has both tumor promoting and tumoricidal effects depending on cancer types (12-16). AUF1 has been suggested to modulate different cancer-related events including apoptosis, tumorigenesis and development (10). However, the role of AUF1 in ESCC progression has not been reported. In the present study, we found that the expression of AUF1 was significantly higher in ESCC tissues than that in normal tissues and tumor adjacent tissues. In this study, we found that AUF1 levels are correlated with tumor stage, indicating that AUF1 may be a predicator of malignant degree, and related with prognosis of the malignancy. We also found that AUF1 silencing resulted in markedly decreased cell proliferation and focus formation and elevated cell apoptosis.

AUF1 has been implicated in regulating the expression of numerous mRNAs containing 3'UTR ARE, including Bcl2, cyclin D1 and SOD1 (10,13,14). However, the regulatory profile of AUF1 may be cell-specific. To comprehensively understand the function of AUF1 in esophageal cancer, we performed mRNA microarray-based screening analysis in Eca-109 cells. Through this analysis, we found that overexpression of AUF1 affected multiple genes, such as Nalcn, UGP2, BEX2 and Prdm16. Among AUF1 affected genes, GCH1 encoded protein is the first and rate-limiting enzyme in tetrahydrobiopterin (BH4) biosynthesis, catalyzing the conversion of GTP into 7,8-dihydroneopterin triphosphate (25). BH4 is an essential element required for nitric oxide (NO) formation by all nitric oxide synthases (NOSs) (26). NO has been reported to modulate many cancer-related events, such as angiogenesis, apoptosis, cell cycle, invasion and metastasis (27), and correlated with the development of several cancers, including breast, cervical, lung, gastric and head and neck cancer (28-33). Therefore, GCH1 is likely to play important roles in cancer progression. Chen *et al* (34) reported that GCH1 promotes cancer angiogenesis *in vitro* and *in vivo*. Consistently, another group demonstrates that GCH1 inhibition reduces tumor growth by: i) direct killing of tumor cells; ii) inhibiting angiogenesis; and iii) enhancing the antitumoral immune response. However, little is known about the functional significance of GCH1 in ESCC cancer. Here, we report that GCH1 was regulated by AUF1 and involved in the proliferation and colony formation in ESCC cells. The function of GCH1 in other aspects and regulatory mechnisms merit further investigation.

In summary, we found that AUF1 expression was significantly increased in ESCC tumor tissues, and that its expression correlated with tumor stage in human ESCC tissue samples. Furthermore, knockdown of AUF1 resulted in markedly decreased proliferation and increased cell apoptosis. AUF1 silencing-affected genes were screened by microarray assay. In particular, we demonstrated that AUF1 regulated the expression of GCH1 through its 3'UTR. Silencing of GCH1 inhibited the growth of ESCC cells. In conclusion, AUF1/GCH1 axis may present a novel potential target in the treatment of ESCC.

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