

# Time-Dependent Decrease of Clusterin as a Potential Cerebrospinal Fluid Biomarker for Drug-Resistant Epilepsy

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**Abstract** Our previous study on proteomic analysis has shown that clusterin (CLU) is significantly decreased in the cerebrospinal fluid (CSF) of patients with epilepsy. Therefore, the present study aimed to confirm CLU concentration reduction in the CSF of patients with drug-resistant epilepsy and drug-responsive epilepsy. Fifty-two patients with epilepsy (23 drug resistance and 29 drug effectivity) and 20 control individuals were recruited. The concentrations of CSF and serum CLU were detected. Moreover, alteration of CLU was detected in the rat hippocampus over time after pilocarpine-induced status epilepticus (SE). Our results showed that human CSF-CLU levels were decreased in patients with both drug-resistant epilepsy and drug-responsive epilepsy compared to controls, and concentration of CSF-CLU was obviously lower in drug-resistant epilepsy than in drug-responsive epilepsy. In the pilocarpine-induced seizure rats, expression of neuronal CLU was gradually decreased in a time-dependent manner from acute phase to chronic phase after the onset of SE. In conclusion, CLU level is decreased in the CSF of human epilepsy and the similar alteration is confirmed in a rat model with epilepsy. Therefore, CLU might contribute to the development of epilepsy and be a potential CSF biomarker for resistant epilepsy.

**Keywords** Epilepsy · Clusterin · Cerebrospinal fluid · Rat model · Resistance

## Introduction

Epilepsy is a common neurological disease affecting approximate 50 million people worldwide. After rational antiepileptic drugs (AEDs) therapy, 20–30 % of epilepsy becomes drug-resistant. Pharmacoresistant epilepsy is a complicated condition in clinical practice, and this condition remains a diagnostic and therapeutic challenge to date. Therefore, requirement to find out useful biomarkers is urgent for early diagnosis of pharmacoresistant epilepsy. It is reported that apoptosis is an important pathophysiological process in the development of epilepsy (Meldrum 2002; Okamoto et al. 2010; Engel et al. 2013; Henshall and Engel 2013); however, the complex signaling molecules contributing to neuronal apoptosis of epilepsy has not been clearly understood to date.

Clusterin (CLU), also named as apolipoprotein J and sulfated glycoprotein 2, is an enigmatic glycoprotein involved in numbers of biological processes including sperm maturation, tissue differentiation, tissue remodeling, membrane recycling, lipid transportation, cell proliferation and death, tumor progression, and neurodegeneration (Blaschuk et al. 1983; Fritz et al. 1983; May and Finch 1992; Bettuzzi 2009). CLU shows a protective effect to brain injury via stabilization of stressed proteins and inhibition of apoptosis (Wiggins et al. 2003). Moreover, CLU is associated with nerve cell death following status epilepticus (SE) (Michel et al. 1992; Schreiber et al. 1993; Dragunow et al. 1995). Therefore, it is reasonable that CLU participates in epilepsy-related apoptosis.

Human *CLU* gene mainly encodes secreted heterodimeric glycoprotein, which is termed as secretory CLU (sCLU). Recent study has indicated that CLU can be secreted into the

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cerebrospinal fluid (CSF) (Suzuki et al. 2002; Nilseid et al. 2006). Therefore, CSF-CLU change can represent the alteration of the brain. A previous study from our laboratory shows that CLU is significantly decreased in the CSF of patients with epilepsy by proteomic analysis (Xiao et al. 2009). We hypothesize that decreased CLU probably attenuate anti-apoptosis effect, thus leads to seizure-induced neuronal death in the brain, and seizures eventually become intractable. Herein, we designed this study to confirm that CSF-CLU is decreased in patients with drug-resistant epilepsy compared to that in patients with drug-responsive epilepsy and controls. Moreover, we also observed decreased CLU level in the hippocampus during the process of epilepsy in pilocarpine-induced rats.

## Methods

### Patients and Samples

A total of 52 patients (28 male, 24 female) were randomly recruited from the epilepsy center of The First Affiliated Hospital, Chongqing Medical University, Chongqing, Southwest China. Patients were diagnosed as idiopathic epilepsy according to the criteria proposed by International League Against Epilepsy in 2001. The patients were divided into two groups: drug-responsive group ( $n=29$ ) and drug-resistant group ( $n=23$ ). In addition, 20 individuals (11 male, 9 female) were used as controls. All controls had no history of neuropsychological diseases and exposure to antiepileptic drugs administration. The informed consents were obtained from all participants.

Four milliliters of CSF and 5 ml venous blood were collected from patients and controls. The total protein and biochemistry of CSF and blood were analyzed, and the remaining CSF was centrifuged at 2,000 rpm for 10 min at 4 °C to separate cell component, and the remaining blood was centrifuged at 3,000 rpm for 15 min to collect the serum. All samples were stored at –80 °C for later analysis.

### Animals and Brain Samples

Adult male Sprague–Dawley rats (age 6–8 weeks, weight 180–230 g) were provided by the animal center of Chongqing Medical University. Rats were housed in individual cages and kept under controlled environmental conditions (22–25 °C, 50–60 % humidity, 12/12-h dark/light cycle) with free access to standard laboratory water and feed for at least 1 week to allow the animals to acclimate.

The experimental rats received lithium chloride (127 mg/kg i.p., Sigma, USA), and pilocarpine (50 mg/kg, i.p., Sigma) was injected after 20 h for inducing animal model of temporal lobe epilepsy (TLE). Atropine sulfate

(1 mg/kg i.p.), which is a muscarinic antagonist, was administered 30 min prior to the pilocarpine or saline injection to reduce the adverse peripheral affects of pilocarpine. Then, rats received repeated intraperitoneal injections of 10 mg/kg pilocarpine until SE (an interval of 30 min for the first and second repeated injections and 10 min for the rest of the repeated injections). Seizure activities were classified as 0–5 stages according to Racine classification (Racine, 1972). Only rats that had exhibited repeated convulsive seizures (stage 4 or 5) were used in the present study. Experimental seizure rats were divided into six subgroups (10 rats per group) stratified by the onset after SE: 24 h, 48 h, 1 week, 2 weeks, 1 month, and 2 months. In addition, 10 control rats were given an i.p. injection of an equal volume of saline instead of pilocarpine.

Brain tissues of experimental rats were dissected under anesthetized with 3.5 % chloral hydrate (1 ml/kg, i.p.). One side of the hippocampus was frozen and stored at –196 °C liquid nitrogen for Western blot analysis. The remaining hippocampus was fixed with 4 % paraformaldehyde solution overnight and then embedded in paraffin for immunohistochemistry and immunofluorescence.

### Ethics

The present study was approved by the ethics committee on human and animal research at Chongqing Medical University.

### Enzyme-Linked Immunosorbent Assays

Human CSF-CLU was determined using Enzyme-linked immunosorbent assays (ELISA), with Human CLU Elisa Kit (Usen Life Science & Technology Company, Wuhan, China). One hundred milliliters of the standard, blank, and samples (CSF samples were diluted 5 times, and serum samples were diluted 10 times according to our preliminary experiments results) in was added in each well and incubated for 2 h at 37 °C covered with plate sealer. The liquid was then removed from each well and 100 ml detection reagent A working solution was added to each well and incubated for 1 h at 37 °C; then, each well was aspirated and washed by filling each well with wash buffer using a multichannel pipettor. The process was repeated three times. After that, 100 ml detection reagent B working solution was added to each well and incubated for 1 h at 37 °C and the aspirate and wash steps were repeated. Then, 90 ml substrate solution was added to each well and incubated for 30 min at 37 °C, protecting from light. To each well, 50 ml stop solution was added if color change does not appear uniform. Finally, the optical density of each well was determined at once, using a microplate reader set to 450 nm on Multiscan Spectrum Microplate Spectrophotometer (Thermo Fisher Scientific, USA).

## Immunohistochemistry

Rat hippocampus sections were deparaffinized in xylene and rehydrated in graded ethanol before staining. Seizure and control sections were run alongside each other in the same immunohistochemistry run. The procedure was performed as follows: endogenous peroxidase activity was blocked with 3 % H<sub>2</sub>O<sub>2</sub> for 15 min. Antigen recovery was performed by heating the sections in 10 mmol/l boiling sodium citrate buffer at pH 6.0 for 10 min. A blocking solution of normal goat serum was then added onto the sections at room temperature for 30 min. The primary antibody (CLU, rabbit anti-rat polyclonal antibody, 1:50 dilution, Beijing Biosynthesis Biotechnology Co., LTD, China) was incubated at 4 °C overnight. Sections were incubated with anti-rabbit secondary antibody for 30 min at 37 °C. Sections were then treated with ABC solution (Zhongshan Golden Bridge Inc., Beijing, China) at 37 °C for 30 min. Immunoreactivity was observed with 3,30-diaminobenzidine (DAB) (Zhongshan Golden Bridge, Inc.) for 5 min. Negative controls were processed simultaneously by replacing the primary antibodies with PBS. A series of 10 random visual fields of each section were obtained using an AX80 microscope (Olympus, Tokyo, Japan), and the images were obtained by TC-FY-2050 pathology system acquisition image and were automatically analyzed by Motic Med 6.0 CMIAS pathology image analysis system (Beihang Motic, Inc., Beijing, China). The computer software determined the intensity of labeling which gave a gray value ranging from 0 (black) to 1.0 (white). Immunohistochemical parameters assessed the mean optical density (OD) which indicated the mean intensity of stain in each pixel and the mean amount of staining material in that area.

## Immunofluorescence

After deparaffinization, rehydration, and antigen retrieval, tissue section were incubated in 0.5 % Triton X-100 for 10 min, and then in normal goat serum for 30 min (Zhongshan Golden Bridge Inc.) The sections were incubated at 4 °C overnight with CLU at the dilution of 1:50 and anti-neuron specific enolase (NSE) antibody (Zhongshan Golden Bridge Inc.) at a dilution of 1:50. After washing in PBS, the sections were incubated in the dark with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:100, Zhongshan Golden Bridge Inc.) and tetramethyl-rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG (1:100, Zhongshan Golden Bridge Inc.) for 3 h. After washing in PBS, the sections were mounted, sealed, and dried overnight. Fluorescent-stained sections were examined by confocal laser scanning microscopy (Leica Microsystems Heidelberg GmbH, Germany), and the images were collected and processed using Olympus Micro image (version 4.0).

## Western Blot

Protein extraction was performed directly according to the manufacturer's routine protocol (Keygenbio, Nanjing, Wuhan, China). Lysate protein concentrations were determined using the bicinchoninic acid (BCA) protein assay (MultiSciences Biotech, Beijing, China). Protein extracts (100 µg per lane) were then added to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5 % spacer gel, 80 V, 30 min; 10 % separation gel, 100 V, 60 min). Protein bands in each gel were electrophoretically transferred to a polyvinylidene fluoride membrane at 250 mA for 1 h. Equivalent protein loading/transfer was confirmed by Ponceau S staining of the membranes. Membranes were incubated at 37 °C for 1 h in 5 % skim milk to block nonspecific binding. Membranes were incubated with polyclonal rabbit anti-rat CLU (1:100, Beijing Biosynthesis Biotechnology Co., LTD) at 4 °C overnight, washed four times with Tris-buffered saline-Tween 20 (TBST) and then incubated with secondary antibody for 1 h at 37 °C. After extensive washing with TBST, membranes were visualized by chemiluminescence with an ECM kit. CCD confocal imaging was used to visualize protein bands. Data were normalized to the amount of β-actin (1:5,000, Zhongshan Golden Bridge Inc.) staining. Protein bands were analyzed using the Gene-Tools 2.0 software.

## Statistical Analysis

Descriptive data were expressed as means ± standard deviation (S.D.). Independent sample *t* test and one-way analysis of variance (ANOVA) were used. In addition, to analyze the correlations of CLU with the age, gender, and seizure duration, we performed multivariate logistic regression analysis. A *P* value less than 5 % was considered as significant. Statistical analysis was performed using SPSS statistics software, version 11.5.

**Table 1** Clinical characteristic of the subjects

	Drug-responsive ( <i>n</i> =29)	Drug-resistant ( <i>n</i> =23)	Control ( <i>n</i> =20)	<i>P</i> value
Age (year)	31.24±13.56	27.83±11.98	36.15±15.82	>0.05
Gender				
Female	14	10	9	>0.05
Male	15	13	11	
Duration of seizures (year)	2.11±0.84	15.68±9.37	Not applicable	<0.05

**Table 2** Clinical data of the patients in the study

Number	Age (year)	Gender	Age of onset (year)	Duration of seizures (year)	AEDs prescribed	AEDs response
1	13	M	10	3	VPA	Effective
2	13	F	12	1	PB	Effective
3	56	M	55	1.5	CBZ	Effective
4	54	M	53	1.25	CBZ	Effective
5	33	M	29	4	VPA	Effective
6	43	M	42	1	TPM	Effective
7	17	M	16	1.33	LEV	Effective
8	60	M	58	2	VPA	Effective
9	48	F	45	2.83	TPM	Effective
10	24	F	23	1.25	VPA	Effective
11	23	F	19	4.17	VPA,CBZ	Effective
12	30	F	29	1	LTG	Effective
13	45	M	43	1.67	VPA	Effective
14	17	M	15	2.08	VPA	Effective
15	24	F	21	3	TPM	Effective
16	23	F	21	1.83	TPM	Effective
17	31	F	28	3	VPA	Effective
18	42	M	40	2	LEV	Effective
19	18	F	16	2	VPA	Effective
20	23	F	20	2.67	VPA	Effective
21	23	F	20	3	TPM	Effective
22	28	F	26	2.25	VPA	Effective
23	42	M	40	2	CBZ	Effective
24	22	F	20	1.75	TPM	Effective
25	46	M	45	1	CBZ	Effective
26	20	M	18	2	VPA	Effective
27	35	M	32	2.58	VPA	Effective
28	37	F	35	1.92	CBZ	Effective
29	16	M	14	2	VPA	Effective
30	41	M	1	40	CBZ,PB,VPA, TPM	Resistant
31	11	M	2	9	VPA,TPM,LTG	Resistant
32	38	M	30	8	CBZ, VPA,TPM	Resistant
33	39	M	9	30	CBZ, VPA,TPM	Resistant
34	24	F	14	10	CBZ,VPA,TPM, LTG	Resistant
35	22	F	8	14	CBZ,VPA,TPM, LEV	Resistant
36	28	M	8	20	VPA,TPM,LEV	Resistant
37	13	F	9	4.25	CBZ, VPA,TPM	Resistant
38	41	M	17	24	VPA,TPM,LEV	Resistant
39	13	F	4	9	VPA,TPM,LTG	Resistant
40	33	M	25	8	CBZ, PB,VPA, TPM	Resistant
41	51	F	30	21	CBZ,VPA,TPM	Resistant
42	11	M	2	9	CBZ,VPA,TPM, LEV	Resistant
43	38	M	20	18	CBZ,VPA,TPM	Resistant
44	26	F	1	25	CBZ, VPA,TPM,PB	Resistant
45	37	F	7	30	VPA,TPM,PB, LEV	Resistant
46	17	M	9	8	CBZ, VPA, TPM, PB	Resistant
47	20	M	2	18	VPA,TPM,PB, LEV	Resistant
48	32	F	28	4	CBZ,VPA,TPM, PB	Resistant

**Table 2** (continued)

Number	Age (year)	Gender	Age of onset (year)	Duration of seizures (year)	AEDs prescribed	AEDs response
49	45	M	39	5.5	CBZ,VPA,TPM, PB	Resistant
50	28	M	12	16	VPA, LTG,TPM,LEV	Resistant
51	15	F	0	15	CBZ,VPA,TPM, LEV	Resistant
52	17	F	2	15	CBZ,VPA,TPM, PB	Resistant

F female, M male, VPA valproate acid, PB phenobarbital, CBZ carbamazepine, TPM topiramate, LTG lamotrigine

**Results**

**Characteristics of Patients and Controls**

The average age of patients was  $29.73 \pm 12.87$  years, ranged from 11 to 60 years. The duration of seizures was significantly higher in patients with drug-resistant epilepsy than that in patients with drug-responsive group ( $15.68 \pm 9.37$  vs.  $11 \pm 0.84$ ,  $P < 0.05$ ). The details of patients were presented in Table 1. Moreover, age and gender of control individuals were matched compared to epilepsy ( $P > 0.05$ ). Tables 1 and 2 summarized the clinical data of all participants.

**CLU Concentration in the CSF of Human Epilepsy**

CSF-total protein was  $0.41 \pm 0.15$  g/l in drug-responsive group,  $0.45 \pm 0.23$  g/l in drug-resistant group, and  $0.47 \pm 0.09$  g/l in controls, respectively. Serum total protein was  $61.66 \pm 4.22$  g/l in drug-responsive group,  $62.35 \pm 3.46$  g/l in drug-resistant group, and  $61.30 \pm 3.80$  g/l in controls, respectively. The total protein of CSF and serum had no significance in drug-responsive patients, drug-resistant patients, and control group ( $P > 0.05$ ).

The concentration of CSF-CLU was  $49.69 \pm 11.11$  ng/ml in drug-resistant group,  $251.83 \pm 93.16$  ng/ml in drug-responsive group, and  $328.10 \pm 140.86$  ng/ml in controls, respectively (Fig. 1). ANOVA analysis showed that there was significantly different among those three groups ( $P < 0.05$ ). Dunnett’s *t* test showed that concentration of CSF-CLU in both drug-effective group and drug-resistant group was lower than that in controls ( $P < 0.05$ ); moreover, CSF-CLU was significantly lower in drug-resistant epilepsy subgroup than in drug-responsive epilepsy subgroup ( $P < 0.05$ ).

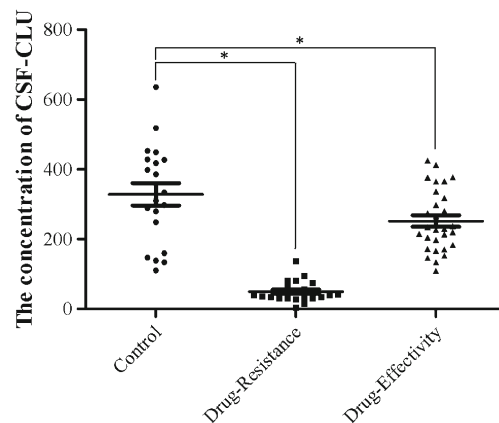
Serum CLU level was  $163.55 \pm 32.89$  ng/ml in drug-resistant group,  $245.74 \pm 48.26$  ng/ml in drug-responsive group, and  $221.46 \pm 191.27$  ng/ml in controls, respectively. One-way ANOVA analysis showed that there were significant differences among three groups ( $P < 0.05$ ). Dunnett’s *t* test showed serum CLU level in drug-resistant group was significant lower than that in drug-responsive group ( $P < 0.05$ ). No statistic difference was found between drug-responsive group and controls ( $P > 0.05$ ).

In addition, logistic regression analysis showed that CSF-CLU concentration had a positive correlation with seizure duration of patients ( $P = 0.0174$ ), but no deviations with age and gender of patients ( $P > 0.05$ ).

**CLU Expression in Rat Brain**

Hippocampus tissues were used for immunohistochemical staining in both model rats and controls. Positive immunoreactivity for the staining of CLU protein was observed in controls and gradually decreased in pilocarpine-induced rats in a time-dependent manner (Fig. 2). The mean optical densities of CLU expression was  $223.81 \pm 45.10$  in control group,  $102.53 \pm 4.31$  in 24 h after SE,  $68.64 \pm 0.78$  in 72 h after SE,  $57.46 \pm 0.24$  in 1 week after SE,  $51.62 \pm 0.30$  in 2 weeks after SE,  $46.47 \pm 3.34$  in 1 month after SE, and  $37.03 \pm 1.82$  in 2 months after SE, respectively ( $P < 0.05$ ). Moreover, immunofluorescence showed that CLU (green) and NSE (red) were co-expressed in neurons, indicating that CLU located in neurons (Fig. 3).

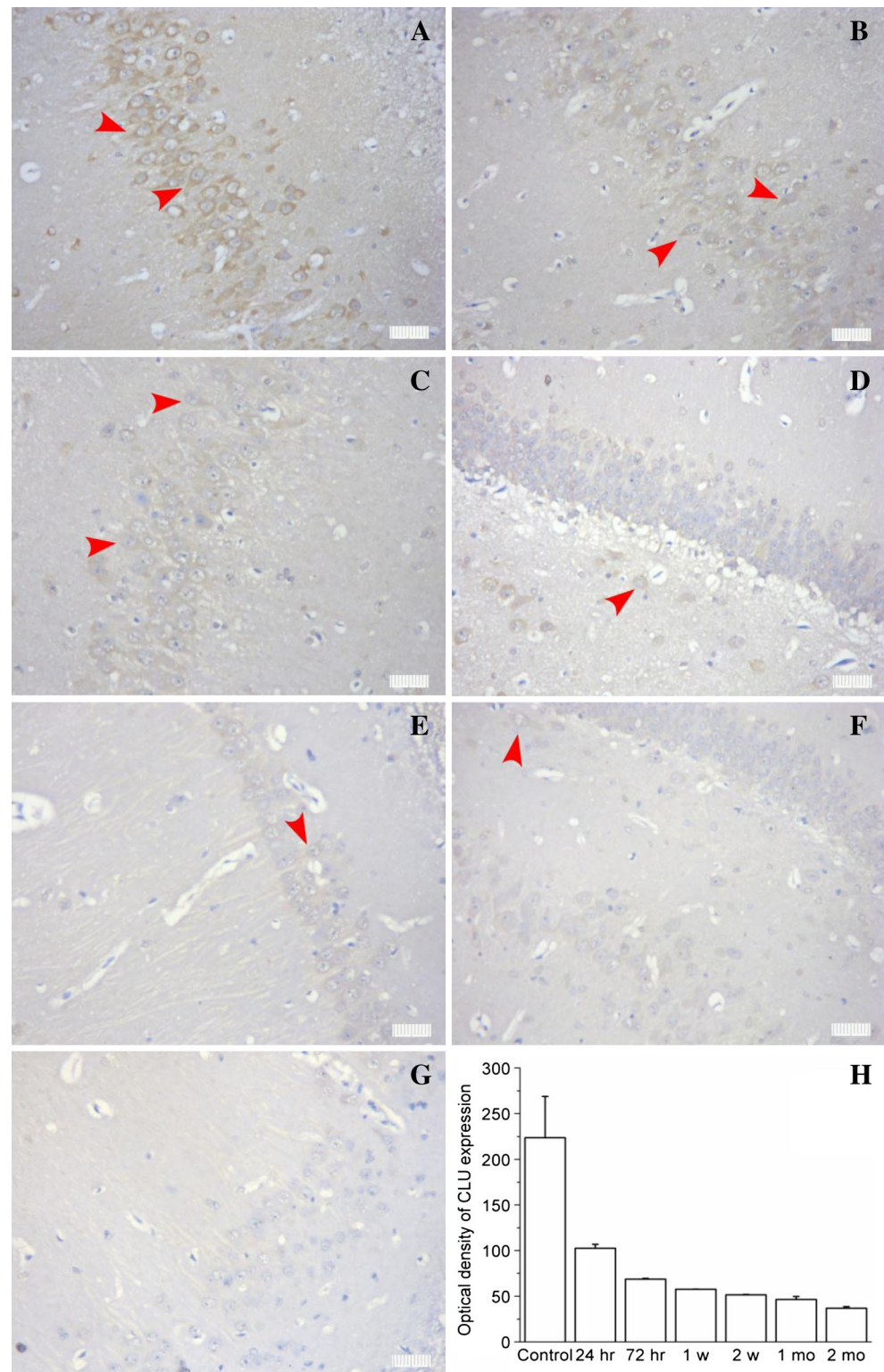
To further confirm the decrease in the immunohistochemical staining of seizure rats with different process of epilepsy, we tested the expression levels of CLU by Western blot. CLU



\* represented that CSF-CLU level in both drug-effective group and drug-resistant group was lower than that in controls ( $P < 0.05$ )

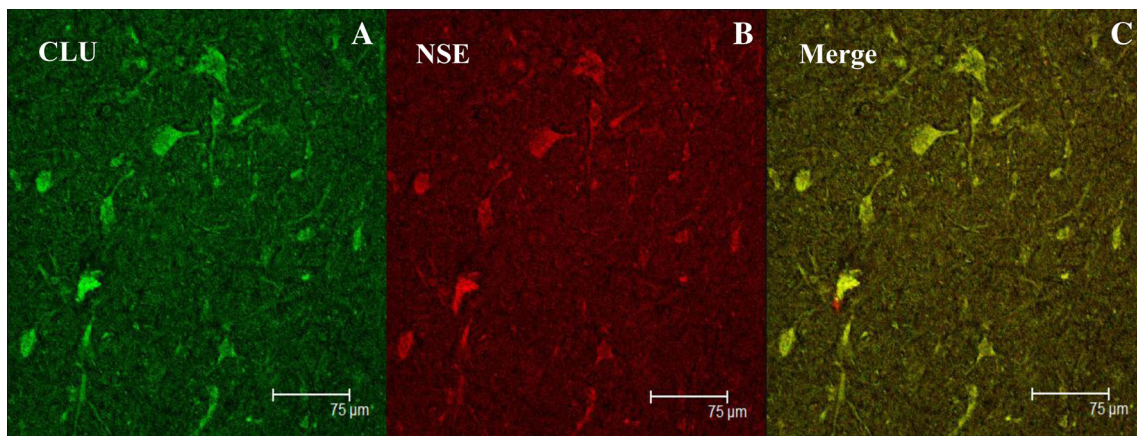
**Fig. 1** CSF-CLU level in epileptic patients is lower than that in control group. The asterisks represented that CSF-CLU level in both drug-effective group and drug-resistant group was lower than that in controls ( $P < 0.05$ )

**Fig. 2** Expression of CLU in hippocampus of rats ( $\times 400$ ). **a–g** Control, 24 h, 72 h, 1 week, 2 weeks, 1 month, and 2 months pilocarpine groups, respectively. (Scale bar: 100  $\mu\text{m}$ ). **h** Quantification of CLU gradually decreased in different time points. Arrow showed the positive expression of CLU



protein was detected by immunoblot as a band at 50 kD. Strong stained bands were presented in control, and band intensity gradually decreased in epileptic rats following duration of seizures (Fig. 4).  $\beta$ -actin band as positive control at 42 kD also was observed correspondingly in every channel.

The relative optical density of CLU was  $1.72 \pm 0.18$  in control group,  $1.16 \pm 0.15$  in 24 h after SE,  $1.31 \pm 0.31$  in 72 h after SE,  $1.14 \pm 0.07$  in 1 week after SE,  $0.47 \pm 0.04$  in 2 weeks after SE,  $0.40 \pm 0.01$  in 1 month after SE, and  $0.42 \pm 0.03$  in 2 months after SE, respectively ( $P < 0.05$ ).



**Fig. 3** CLU expressed in neuronal cells of rat hippocampus. Immunostaining of rat hippocampus section with **a** anti-CLU (indicated as *green*), **b** neuronal marker anti-NSE (indicated as *red*), and **c** merged image (indicated as *yellow*)

**Discussion**

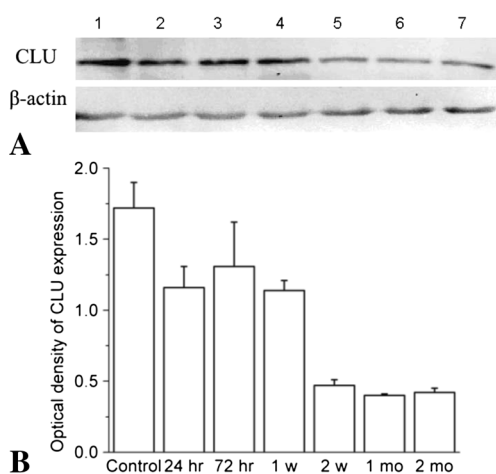
The present study indicates that CLU concentration is decreased in the CSF of patients with epilepsy, and decreased CSF-CLU is more obvious in subgroup of drug-resistant epilepsy than that in drug-responsive subgroup. The time-dependent decrease of CLU protein is confirmed in the hippocampus of epileptic rat models. Our results shows that approximately 85 % decrease of CSF-CLU in patients with resistant epilepsy but only 23 % reduce in patients with drug-responsive epilepsy compared to controls, revealing more marked reduce of CSF-CLU is a potential CSF biomarker for patients with resistant epilepsy.

Human *CLU* gene, including nine exons, is located at 8p21-p12 on chromosome 8. The CLU protein, described as a glycoprotein, is divided as a nuclear form (nCLU) and a secretory form (sCLU). In general, the two forms perform dual role on apoptosis. nCLU shows a pro-apoptosis effect

via driving the fate of cells and is usually associated to cell death (Bettuzzi and Rizzi 2009; Kim and Choi 2011). sCLU shows an anti-apoptosis function via prevention from lipid oxidation-associated cell damage, removal of dead cell remnants in tissues, and clearance of harmful extracellular molecules (Zhang et al. 2005; Klock et al. 2009). Recent data show that CLU involves in numbers of neurological diseases, including traumatic brain injury, stroke, Alzheimer’s disease, Parkinson’s disease, prion protein, multiple sclerosis, and retinopathy (Charnay et al. 2012). Moreover, CLU could act as drug resistance factor against certain cytotoxic agents that induce apoptosis in cancer (Park et al. 2013). Therefore, CLU might play a role in resistant epilepsy since epilepsy has apoptosis mechanism. A recent study indicates that prolonged seizures increase the expression of nCLU in kainic acid-induced mice, indicating that pro-apoptosis effect of nCLU enhances in epilepsy (Kim et al. 2012). Our study shows that CLU (mainly sCLU) levels in both human and animal with epilepsy decrease, indicating that anti-apoptosis of sCLU attenuates in epilepsy. Thus, unbalance of nCLU and sCLU participates in the apoptosis process of epilepsy.

Our study shows that human CSF-CLU levels decrease in patients with epilepsy compared to controls, suggesting that cytoprotective effects of sCLU are attenuated in response to epilepsy and seizures. Moreover, CLU is markedly decreased in the CSF of patients with drug-resistant epilepsy rather than that in patients with drug-responsive epilepsy; thus, apoptosis process becomes probably stronger in drug-resistant epilepsy.

In the present study, patients with drug-resistant epilepsy have significant longer duration of disease compared to patients with drug-responsive epilepsy, and CSF-CLU concentration has a correlation with seizure duration. This phenomenon indicates that severity of CSF-CLU decrease is associated with disease duration. Furthermore, time-dependent alteration of CLU is also confirmed from acute phase to chronic phase following of SE onset in animal rats with epilepsy. Therefore, we suppose that repeated seizures and disease



**Fig. 4** Western blot analysis of CLU in hippocampus in seizure rats. **a** Samples 1–7 represented control, 24 h, 72 h, 1 week, 2 weeks, 1 month, and 2 months groups, respectively. **b** CLU decreased in rats following SE onset at different time points

progression cause attenuated expression of CLU, and anti-apoptosis effect of CLU permanently attenuates in a time-dependent manner, further leading to neuronal network recombination, which makes seizure become drug resistant. The time-dependent decrease of CLU expression indicates that CLU alteration is related to seizures. We thus think that CLU participates in the development of epilepsy.

In the present study, we assume that downregulation of CLU during the process of epilepsy might promote epileptogenesis via modulating apoptosis-associated molecular signaling, such as endoplasmic reticulum (ER) stress, unfolded proteins, and molecular chaperone (Nizard et al. 2007; Wilson and Easterbrook-Smith 2000; Poon et al. 2000). Seizure could be regarded as a stress to attenuate CLU expression, causing CLU relocated into cytosol through ER-associated protein degradation pathway, and ER stress can cause accumulating unfolded proteins; CLU binds to hydrophobic regions of unfolded proteins via an ATP-independent mechanism, which can stop protein aggregation and precipitation; the attenuation of CLU would increase protein precipitation. The process further contributes to neuronal apoptosis and eventually leads to epilepsy. Moreover, CLU acts as an extracellular molecular chaperone; overexpression of CLU contributes to protective effect against certain type of cytotoxic agents that induce apoptosis; thus, the decrease of CLU would impair the protection and promote apoptosis, which deteriorates epilepsy. However, CLU has been functionally associated with numerous non-apoptosis binding molecules, including Apo A-I, lipids, paraoxonase, complement components, immunoglobulin,  $\beta$ -amyloid peptide, glycoprotein H, glutathione S-transferase, Ku86 and gp330/megalin receptor (Park et al. 2013), suggesting multiple mechanisms of CLU might trigger the development of epilepsy and contributes to resistant epilepsy.

We have to acknowledge a few limitations in this study. Firstly, no normative reference value is concluded to distinguish drug-resistant epilepsy and drug-responsive epilepsy due to the limitation of sample size; therefore, the further study will be performed to enlarge the samples to confirm the reference value for distinguish drug-resistant epilepsy from drug-responsive epilepsy. Secondly, the role of serum CLU change is different compared to CSF-CLU change; we thus think that peripheral CLU cannot predict the change of central environment. Therefore, blood CLU cannot be used in predicting resistant epilepsy. Thirdly, evidence has shown that epilepsy has blood-brain barrier (BBB) dysfunction (Oby and Janigro 2006; Seifert et al. 2006). However, no BBB experiments has been design in the present study; therefore, we do not know whether CLU have some effect upon the BBB function since it has a protective effect on the blood-retinal barrier (Kim et al. 2010).

In conclusion, CLU level is decreased in the CSF of human epilepsy and in the hippocampus of rat model. Decreased level

of CLU is associated with repeated seizures and contributes to the development of epilepsy. Therefore, CLU might be a potential CSF biomarker for human resistant epilepsy.

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**Conflict of Interest** The authors have no conflicts of interest to declare.

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