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Kininogen Level in the Cerebrospinal Fluid May Be a Potential Biomarker for Predicting Epileptogenesis

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Hippocampus and Insula Are Targets in Epileptic Patients With Glutamic Acid Decarboxylase Antibodies

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Editorial: Burden of Illness in People With Epilepsy: From Population-Based Studies to Precision Medicine

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surviving neuron populations to generate abnormal, synchronous, and recurring epileptiform discharges that produce focal or generalized behavioral seizures (4). Epileptogenesis period refers to a time period between the insult and the occurrence of the first unprovoked seizure (5). As clinicians are unable to figure out which patient will develop spontaneous epilepsy secondary to the aforementioned brain insults, antiepileptic treatment usually starts after recurrent seizure attacks, which may occur weeks, months, or years after primary brain insult. Therefore, if effective nontraumatic biomarkers for epilepsy can be evaluated prior to significant seizure attacks, it can reduce the risk of recurrent seizure attacks and improve the prognosis. Thus, in recent years, the exploration for biomarkers of epileptogenesis has become one of the major focuses in epilepsy research field (6, 7). The expression levels of certain miRNAs (8) and mRNAs were found to be upregulated with the use of peripheral blood of epileptic animals as a sample (9, 10). However, to what extent the changes in peripheral blood reflect the changes in brain network function remains unknown.

Compared with peripheral blood, the cerebrospinal fluid (CSF) better reflects pathological changes in the brain. Lumber puncture has long been proved to be a safe and essential

Preparation of Tryptic Digests

Each sample of 15 μl CSF was diluted using 55 μl 100 mM NH4HCO3 and 10 μl 100 mM DTT at 60°C for 1 h. After incubation, 10 μl of 450 mM (83.23 mg/ul) was added for carboxymethylation, and the sample was allowed to incubated for 30 min in dark. Protein digestions were conducted over with trypsin (100 ng/μl) in a 1:20 trypsin-to-protein mass-ratio. Digestion was performed overnight at 37°C and further incubated at 56°C for another 20 min in the next day morning. The tryptic peptides were dried using Savant SpeedVac (Thermo Scientific) and resolved in 20 μl 0.1% formic acid. Sample was then desalted and purified individually by 10 μl ZipTip pipette tip system (Millipore). Protein attached to the resin in Ziptip was dissolved in 20 μl eluting buffer (80% acetonitrile + 0.1% formic acid+ H_2O) and dried by Savant Speedvac. Finally, sample was reconstituted to 40 μl (2% acetonitrile + 0.1% formic acid + H_2O) and 20 μl was analyzed by Nano LC-MS/MS.

Nano LC-MS/MS

Each sample was analyzed using an LC system (Nano Pump, Ultimate 3000, Dionex, Thermofisher) coupled with an ESI-Q-TOF mass spectrometer (maxis,Impact, Bruker Daltonik, Germany). Each peptide sample was separated using a solvent system with solvent A consisting of 99.9% water and 0.1% formic acid, and solvent B consisting of 99.9% acetonitrile and 0.1% formic acid. The peptides were eluted with gradients 2–20% B in 75 min, 20–80% B in 15 min, 80% hold for 15 min, 2% hold for 15 min with a constant flow rate of 350 nl/min. The LC setup was coupled online to a Q-TOF using a nano-ESI source (Bruker Daltonik, Germany) in data dependent acquisition mode (m/z 350∼1,500). The Source Capillary was set at 2400 v. the flow and temperature of dry gas was 2.0 1/min and 120°C respectively. The mass spectrometer was set as one full MS scan followed by 10 MS/MS scans on the 10 most intense ions from the MS spectrum.

MS Data Analysis

Tandem mass spectra were processed using PEAKS Studio version 41533(e)-0.0567516(1)-0.21505730562R4189.129(41533(e)-0.056(l)0.0541704(l254(n)92(H)303.129(41533(e)-0.05670.246346(e)-0.061909
rst-0.246346(r)0.140585(o)00.160577())-337.707(a)0.305676(n)0.219288/R80.305676(950.1392886(0.41792886(n)0-337.7761(41.65349(a)-00.305676(n)0.21
syes

with the clinical manifestation of encephalopathy: consciousness changes lasted more than 24 h, the appearance of indifference, irritability or personality and behavior changes. (2) two or more of the following manifestations: (a) fever ($\geq$38°C); (b) seizures; (c) focal neurological signs; (d) CSF leukocytes $\geq$ 5/per high magnification; (e) electroencephalography(EEG) abnormalities were consistent with encephalitis changes; (f) neuroimaging suggested changes in encephalitis. (3) no epilepsy history.

As CSF tests are essential in encephalitis diagnosis, the collection of CSF is easy and harmless to patients. The CSF of last lumbar puncture before discharge of patients was collected. At that time, encephalitis was well-controlled after proper therapy. Routine tests of CSF, including cell number, protein level, and glucose, recovered to normal, and the patient was ready for discharge. The acute phase of encephalitis was over, yet spontaneous recurrent symptomatic epilepsy did not appear in most patients. This timing, "the postacute phase," was approximate to what was defined as epileptogenesis period in a chronic epilepsy animal model (21). The CSF was centrifuged (3,000 g, 30 min, 4°C), and the supernatants were finally aliquoted and frozen (−80°C) for subsequent analysis.

Patients were followed up for 2 years. Symptomatic epilepsy secondary to encephalitis was diagnosed if unprovoked epileptic seizure appeared during follow-up, regardless of seizure occurrence during acute phase.

Statistical Analysis

Data were expressed as means ± standard error of the mean (SEM). Statistical analysis was conducted using the -test or one-way analysis of variance (ANOVA) with a significance level of < 0.05.

RESULTS

Screening of Proteins in the CSF of SD Rats That Changed During Early Epileptogenesis

Among the 53 rats in the LiCl-Pilo group, 28 met the standards defined by SE. Five rats died and 23 survived 5 days after SE. After CSF collection, nine (9/23) rats died. One rat was excluded because of blood contamination of CSF. The numbers of rats in all three groups enrolled in CSF collection are shown in **Table 1**.

The 13 rats in LiCl-Pilo group were video monitored 6 weeks after SE for 3–6 days. A total of 66 attacks of Racine 2 or more serious seizures were observed. The average seizure frequency was 1.25 ± 1.14 per day. The numbers of epileptic attacks (Racine 2 or severer) during video monitoring in each rat are displayed

TABLE 1 | Numbers of rats enrolled in CSF collection (*n*).

b8F hnkeonic-0	245057(t)-5	909(k5)-0	06172(l)-4
	04988(h)-0	420466(e)-s	

FIGURE 1 | Proteomic analysis of CSF in SD rats 5 days after SE. **(A)** The concentration of total proteins in the Naïve, LiCl and LiCl-Pilo groups. **(B)** The fold changes in proteins differentially expressed in the CSF of the three groups. The protein in the naïve group was normalized to 1.0. **(C,D)** Results of proteomics analysis.

= 0.031, one-way ANOVA) and naïve groups ($n = 7$, 0.21 ± 0.3 μg/ml) (**$p = 0.001$, one-way ANOVA) (**Figure 2A**). Similar to the result of proteomics, the ratio of KNG in the three groups was 1:4.67:12.43 (naïve:LiCl:LiCl-Pilo).

The level of KNG in the hippocampus was examined using Western blotting and in peripheral blood using ELISA technique to explore the source of KNG in CSF in another group of rats. In the hippocampus, the level of KNG was significantly higher in the LiCl-Pilo group ($n = 4$, 4.42 ± 0.84 fold) than in the LiCl ($n = 4$, 2.03 ± 0.76 fold) ($p = 0.002$, one-way ANOVA) and naïve groups ($n = 4$) ($p < 0.001$, one-way ANOVA) (**Figure 2B**, the original Western blotting image can be accessed in **Supplementary Material**). In contrast, the serum expression levels of KNG in the LiCl-Pilo group was not significantly different from that in the LiCl group, although the level of KNG in the LiCl group was significantly higher than that in the naïve group (**Figure 2C**; $n = 4$ for each group, LiCl-Pilo 486.12 ± 14.35 μg/ml; LiCl, 559.38 ± 158.11 μg/ml; naïve 158.72 ± 7.92 μg/ml). The increased CSF level of KNG was concomitant with enhanced expression in the hippocampus but not in peripheral blood, indicating that this upregulation was due to an increased synthesis in brain tissue rather than peripheral synthesis.

Correlation of Postacute Phase KNG Level in the CSF and Symptomatic Epilepsy in Patients With Encephalitis

A total of 12 patients with encephalitis were further enrolled from December 2013 to April 2014 to verify whether the increase in the KNG level in the CSF was related to epileptogenesis in humans. The clinical manifestations and laboratory examinations of encephalitis patients are shown in **Table 3**. Five patients (4 females, 1 male) of functional disorder were enrolled as control,

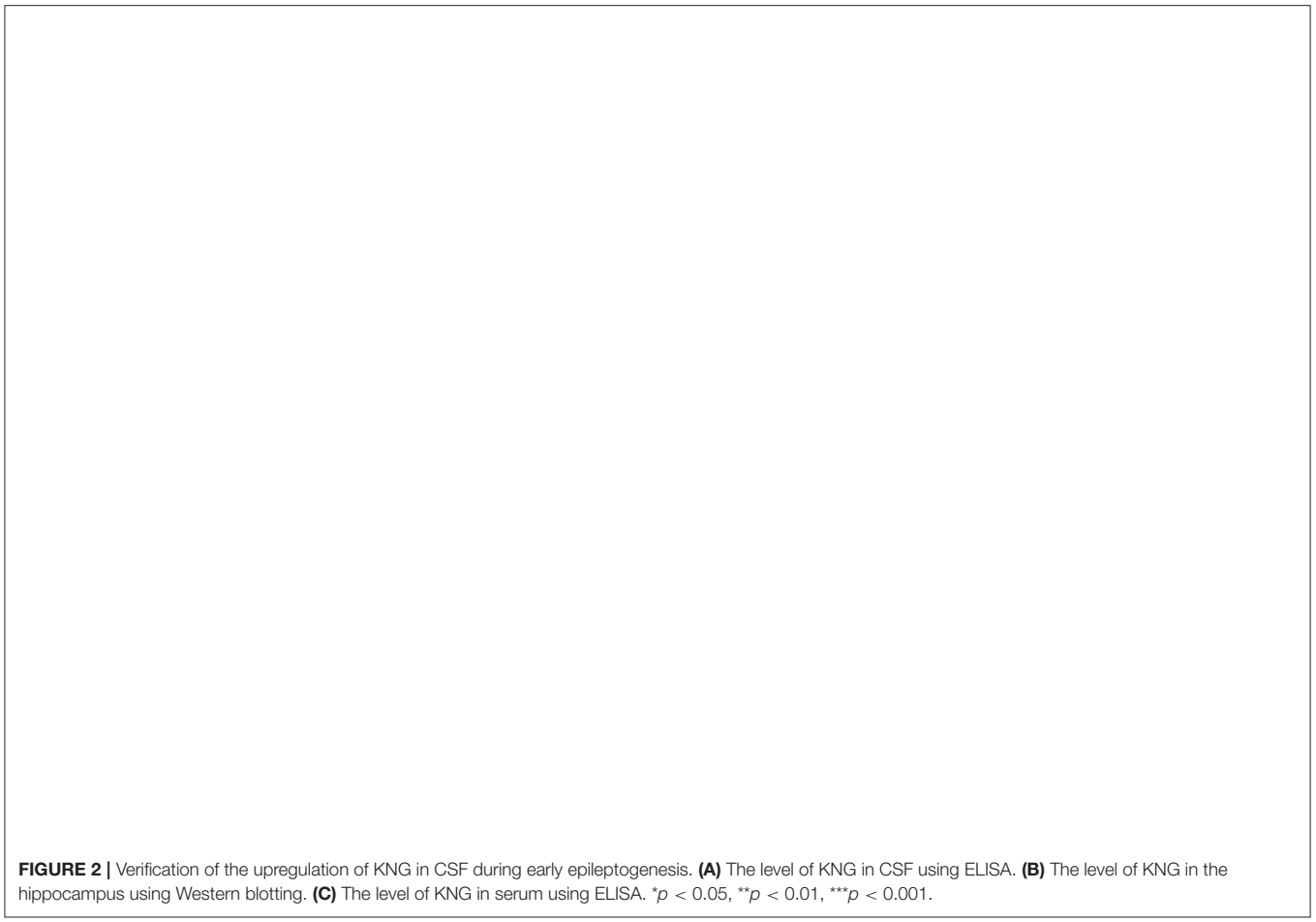

FIGURE 2 | Verification of the upregulation of KNG in CSF during early epileptogenesis. **(A)** The level of KNG in CSF using ELISA. **(B)** The level of KNG in the hippocampus using Western blotting. **(C)** The level of KNG in serum using ELISA. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

including 3 tension-type headache, 1 depression disorder and 1 schizophrenia patients, aged 19-39 years old.

The Level of KNG in CSF and Serum of Encephalitis Patients

As shown in **Figure 3A**, the level of KNG in CSF of encephalitis patients ($1.89 \pm 0.22\,\mu g/ml$, $n = 12$) was significantly higher than that in controls ($1.07 \pm 0.09\,\mu g/ml$, $n = 5$) ($^{**}p < 0.01$, -test). However, the level of KNG in serum of encephalitis patients ($693.55 \pm 46.21\,\mu g/ml$, $n = 12$) was not significantly different than that in controls ($709.68 \pm 35.30\,\mu g/ml$, $n = 5$) ($p > 0.05$, -test) (**Figure 3B**).

The correlation of serum and CSF KNG levels was higher in patients with encephalitis (Pearson coefficient 0.562, $p = 0.057$) than in controls (Pearson coefficient 0.138, $p = 0.825$), but both failed to showed significance (**Figures 3C,D**), suggesting that the upregulated KNG level in the CSF was not likely due to increased synthesis in peripheral tissues, in accordance with the finding in the study on rats.

The Correlation Between Early Epileptogenesis Period CSF and Serum KNG Level and Acute Phase Symptomatic Seizures

Of the 12 patients with encephalitis, seven had symptomatic seizures during acute phase. Thus, the next question was whether the occurrence of acute phase symptomatic seizure had an impact on the increased CSF KNG level in the postacute phase. As shown in **Figure 4A**, the KNG level in CSF of two groups of encephalitis patients were not significantly different with each other ($p = 0.962$, one-way ANOVA), though higher than in controls($^{*}p = 0.029$, one-way ANOVA), indicating that the increased CSF KNG level in early epileptogensis period did not depend on the occurrence of seizures during acute phase. On the contrary, the serum KNG level in all three groups was not significantly different (**Figure 4B**. $p > 0.05$, one-way ANOVA).

The Correlation Between Early Epileptogenesis Period CSF's KNG Level and Symptomatic Epilepsy in 2 Years Follow-Up

This study next compared the CSF KNG levels in controls and encephalitis patients with and without unprovked epilepsy in the 2-year follow-up to verify whether the development of secondary epilepsy after encephalitis was related to the KNG level in the CSF during the postacute phase. As shown in **Figure 5A**, the KNG level in the CSF was significantly higher in patients with secondary epilepsy ($n = 5$, $2.39 \pm 0.29\,\mu g/ml$) than in patients without secondary epilepsy ($n = 7$, $1.52 \pm 0.23\,\mu g/ml$) ($p = 0.046$, one-way ANOVA) or controls ($n = 5$, $1.07 \pm 0.09\,\mu g/ml$) ($p = 0.005$, one-way ANOVA). On the contrary, the KNG level

TABLE 3 | Clinical manifestations and laboratory test results of patients with encephalitis.

Patient number	Gender	Age	Encephalopathy	Fever	Seizure	Neurology signs	Elevated CSF leukocytes	EEG	Neuro-imaging	Diagnosis	SRS in follow-up
1	F	27	+	+	GTCS, Myocl, SPS, PS	+	+	Diffuse abnormity	–	Anti-NMDAR encephalitis	SPS, CPS
2	M	36	+	+	–	–	+	–	Bilateral frontal and temporal lobe lesions	Bacterial meningoe-ncephalitis	SPS
3	M	49	+	+	–	–	+	–	–	Viral encephalitis	–
4	M	17	+	+	GTCS	–	+	–	–	Viral encephalitis	–
5	M	36	+	+	GTCS	–	+	Diffuse abnormity	Bilateral temporal and occipital lobe lesion with enhancement	Viral encephalitis	–
6	M	22	+	+	–	+	–	Diffuse abnormity	–	Viral encephalitis	–
7	F	24	+	+	GTCS, SPS, CPS	+	+	Diffuse abnormity	Bilateral frontal, temporal lobe lesion	Viral encephalitis	GTCS,SPS,CPS
8	M	77	+	+	SPS, Myocl	–	+	Diffuse abnormity	–	Viral encephalitis	SPS
9	M	28	+	+	GTCS, Myocl, SPS, CPS	–	+	Diffuse abnormity	–	Viral encephalitis	GTCS,Myocl,SPS, CPS,AB
10	F	46	+	+	–	–	+	Diffuse abnormity	–	Viral encephalitis	–
11	M	36	+	+	GTCS	–	+	–	–	Viral encephalitis	–
12	M	32	+	+	–	–	+	–	–	Viral encephalitis	–

SPS, single partial seizure; CPS, complex partial seizure; GTCS, generalized tonic-clonic seizure; Myocl, Myoclonic; AB, absence attack.

FIGURE 3 | CSF and serum KNG protein level of encephalitis patients and controls. **(A)** The level of KNG in CSF of encephalitis and control patients. **(B)** The level of KNG in serum of encephalitis and control patients. **(C)** The correlation of serum and CSF KNG levels in encephalitis patients. **(D)** The correlation of serum and CSF KNG levels in control patients. $^{**}p < 0.01$.

FIGURE 4 | Correlation between acute symptomatic seizure and KNG protein level in post-acute phase CSF/ serum. **(A)** The level of KNG in CSF of control and encephalitis patients with and without acute phase symptomatic seizure. **(B)** The level of KNG in serum of control and encephalitis patients with and without acute phase symptomatic seizure. $^{*}p < 0.05$.

FIGURE 5 | Correlation between post-acute phase CSF and serum KNG protein level and symptomatic epilepsy in 2 years. **(A)** The level of KNG in CSF of control and encephalitis patients with and without symptomatic epilepsy. **(B)** The level of KNG in serum of control and encephalitis patients with and without symptomatic epilepsy. $^{*}p < 0.05$, $^{**}p < 0.01$.

in serum was not significantly different among the three groups (**Figure 5B**). Taken together, the increased KNG level in the CSF in the postacute phase was related to unprovked epilepsy during the 2-year follow-up, but not related to acute phase seizures.

DISCUSSION

Increasing clinical evidences indicated that CSF proteins could be potential markers of CNS diseases, such as CSF 14-3-3 protein for Crearzfeldt-Jakob disease (27) and Aβ for Alzheimer's disease (28). Our results showed that CSF KNG level was elevated during early epileptogensis period both in LiCl-Pilo epilepsy model rats and in encephalitis patients who developed epilepsy in the 2-year follow-up period. These findings provided evidence that the elevation of KNG in the CSF might predict epileptogenesis. This was the first study based on both experimental and clinical observations showing that a protein in the CSF might serve as a potential biomarker of epileptogenesis.

Use of CSF to Explore Biomarkers of Epileptogenesis

In this study, about 60 μL of CSF was collected from each model rat and analyzed using a label-free LC-ESI-Q-TOF-MS/MS. CSF studies in epilepsy animal models are rare because CSF collection is not as easy as in patients. A typical yield of CSF is 60 μL for an adult rat and 10 μL for a mouse, in some cases with blood contamination. Compared with serum and brain tissues, the CSF has exceedingly low protein content (about 0.8–1 mg/mL in the present study), influencing the choice of proteomics methods. Various methods of proteomic analysis are used to analyze rodent CSF, including isobaric tags for relative and absolute quantitation (iTRAQ), a method widely used owing to its high sensitivity and relatively satisfactory reproducibility. However, an iTRAQ test usually requires at least 50 μL of sample with 5 mg/mL protein concentration, and can only run 8–12 samples in one test. On the contrary, the label-free proteomic analysis uses spectral counting, which is a frequency measurement that uses tandem mass spectrometry (MS/MS) counts of identified peptides as the metric to enable protein quantitation, hence requiring much less sample volume, lower protein concentration, and no additional sample preparation. Using label-free proteomics method, changes in protein expression were analyzed in the CSF of each rat individually in this study.

The timing of CSF collection is crucial. In a LiCl-Pilo chronic epilepsy rat model, the pilocarpine-induced SE works as a severe brain insult, causing recurrent seizures 7 days on average after the onset of SE. The earliest, first unprovoked seizure reported is 5 or 6 days after the SE (29–31). Therefore, the CSF was collected 5 days after the onset of SE in this study to ensure that sample collection was within the epileptogenesis period.

No strictly defined epileptogenesis period exists for patients with encephalitis with concomitant secondary epilepsy. The period between inflammation process and first unprovoked seizure can be regarded as epileptogenesis period. In this study, we collected the CSF right after inflammation of encephalitis had been controlled. In the acute phase, seizures can be displayed as one of the symptoms in some patients with encephalitis. However, whether it should be regarded as secondary epilepsy is difficult to ascertain. International League Against Epilepsy (ILAE) once defined unprovoked seizure in epidemiologic studies as one occurred >7 days after acute CNS infection (32). In this study, only epileptic attacks that occurred during the 2-year follow-up were regarded as unprovoked epilepsy. The similar CSF KNG level of the postacute phase between encephalitis patients with or without an acute phase seizure (**Figure 4A**) indicated that the elevation of CSF KNG did not depend on the occurrence of a symptomatic seizure.

The CSF contains more than 60% of albumin and several other high-abundance proteins (33, 34). Therefore, some CSF studies reported that depletion of high-abundance proteins helped unmask low-abundance proteins of interest in proteomics (18). In our pilot study, avian polyclonal immunoglobulin Y antibodies (Seppro Rat, sep130, Sigma) were used prior to the proteomic study to remove high-abundance proteins from CSF, including albumin, IgG, fibrinogen, transferrin, IgM, haptoglobin, and alpha-1-antitrypsin. However, it failed to reveal more proteins

AUTHOR CONTRIBUTIONS

JZ was involved in study design, data acquisition, data analysis and interpretation, and drafting of the manuscript. XW and LH were responsible for patient enrollment and follow-up. JL and YK helped JZ with collecting rats' CSF and hippocampus samples. SL and QL were involved in study design, data analysis and interpretation, drafting of the manuscript, study supervision, and critical revision of the manuscript for important intellectual content.

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