C1Q/TNF-related protein 4 expression correlates with herpes simplex encephalitis progression

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Background: Herpes simplex encephalitis (HSE), an acute inflammatory disease of the central nervous system is caused by the herpes simplex virus infection. HSE occurs at any age, and it is often accompanied by high mortality and neurological dysfunction. The C1Q/TNF-related protein (CTRP) family, usually contains a homotrimeric structure, which comprises the N-terminal signal peptide and the C-terminal C1q globular domain. It has been demonstrated that CTRPs play pivotal roles in the inflammation process. CTRP4 is a member of the CTRP family and contains two C1q globular domains. Moreover, evidence shows that the recombinant human CTRP4 (rhCTRP4) protein exerts satisfactory anti-inflammatory effects in experimental colitis models via the NF-κB pathway. However, its role in inflammation-related neurological diseases remains unknown.

Methods: The purpose of this study is to evaluate the expression of CTRP4 and its correlation with HSE progression. We determined the serum CTRP4 levels in a normal brain, tuberculous meningitis (TBM), bacterial meningitis (BM) and HSE.

Results: We found that compared to a normal brain, TBM and BM, CTRP4 was significantly increased in HSE. Moreover, in the course of HSE, serum interleukin (IL-6) and necrosis factor-α (TNF-α) were also increased and were closely associated with CTRP4 expression. CTRP4 expression was examined by immunohistochemistry (IHC) in the normal control brain tissues, HSE, TBM and BM brain tissues. High positively expression of CTRP4 was found in HSE. In the normal brain tissue, TBM, and BM brain tissues, CTRP4 showed a weak expression. In the clinical evaluation, CTRP4 expression correlated closely with an ascending stage of the disease [mini-mental state examination (MMSE) evaluation, MRI imaging).

Conclusions: Our findings suggest that CTRP4 is highly expressed in HSE and is closely related to the progression of HSE. Thus, CTRP4 may serve as a potential severity index for HSE and targeting CTRP4 might be a promising therapeutic strategy against HSE.

Keywords: C1Q/TNF-related protein 4 (CTRP4); herpes simplex encephalitis (HSE); correlation; mini-mental state examination (MMSE)

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Introduction

Herpes simplex encephalitis (HSE) is associated with high mortality, having about a 70% mortality in untreated patients, and a 30% mortality even in the early stages of adequate-antiviral-agent-treated patients (1-3). The infection mainly undermines the temporal and frontal lobes or limbic system. Pathological progress includes edema, hemorrhage, neuronal degeneration, and necrosis in the brain parenchyma (4). Additionally, HSE survivors are often accompanied by severe and permanent neurological damage and focal neurological deficits, such as various degrees of cognitive, memory, and behavioral deficits (5).

Herpes simplex virus is classified as herpes simplex virus 1 and herpes simplex virus 2. Most HSE cases are caused by herpes simplex virus 1 (90%) (6). The primary infection of HSV-1 is often located in the oropharynx, especially gingivostomatitis (7). HSV-1 can cause lip herpes, herpetic keratitis, herpetic dermatitis, and encephalitis. HSE occurs at any age. There are 1 to 3 million people with HSE and it accounts for 5–10% of the viral encephalitis cases in the USA (8).

The mechanisms of HSE pathogenesis has not been fully understood. Studies have shown both viral infection and brain damage is caused by the progression of HSE (9). The innate immune response to the virus is a double-edged sword. On the one hand, the immune response activates the anti-inflammatory system which protects the nervous system (10). On the other hand, nerve damage induced by the excessive immune response is the key reason for disability and death (11,12). There are many pro-inflammatory factors involved in the progression of HSE such as IL-6 and TNF-α. In a mice model, C1Q/TNF-related protein 4 (CTRP4) concentration in peripheral blood was highly consistent with the concentration in brain tissue homogenate levels (13). CTRP4 plays an important role in innate immune response (14), and CTRP4 has been demonstrated to be anti-inflammatory in the colitis model. Evidence has shown that CTRP4 is closely related to inflammatory cytokine through effecting the STAT3 and NF-kB signal pathways (15). However, the function of CTRP4 in HSE is still unclear.

In the present study, we hypothesized that CTRP4 plays an important role in HSE progression. CTRP4 was found highly expressed in HSE by bioinformatics analysis. Using ELISA, we then found that the concentration of CTRP4, IL-6, and TNF-α was highly detected in the HSE serum when compared to others; IHC stained CTRP4 in normal brain, HSE, tuberculous meningitis (TBM), and bacterial meningitis (BM) brain tissues. Mini-mental state examination (MMSE) clinical scores and MRI imaging was evaluated using the different stages of the HSE patients. Data showed a similar tendency to the previous studies. These findings suggest that CTRP4 might act as a potential disease severity index for HSE.

Methods

Ethics statement

This study was approved by the Ethics Committee of Beijing Tiantan Hospital, Beijing Ditan Hospital, and Capital Medical University (NO. KYSQ 2019-011-01). Informed consent was obtained from all patients, and the study was conducted following the ethical standards of the Helsinki Declaration.

Patients sample collection

Serum was obtained from 30 normal donors, 15 HSE patients, 5 BM patients, and 6 TBM patients. One case of HSE had cerebral hemorrhage and cerebral palsy. The patient received a craniotomy and necrotic brain tissue was removed to study the pathology.

There was 1 case of bacterial encephalitis, 1 case of TBM and 1 sample of normal brain tissue which came the from donors. After individual informed consents were obtained from patients and normal persons, venous blood was collected in sterile tubes. 100 μL of blood sample was used for determining CTRP4, IL-6, and TNF-α with ELISA.

Bioinformatics analysis

The gene expression data of GSE51040 were downloaded. We analyzed 23 mice data from hippocampi infected with HSE and 23 normal mice. The gene expression data of GSE6509 were downloaded. We analyzed 20 cases of LPS-induced bacterial encephalitis mice and 9 normal mice. These gene expression data of GSE23074/GSE29507 were also downloaded. We analyzed 10 patients with tuberculous encephalitis and 4 normal persons.

Enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected and naturally coagulated at room temperature for 20 minutes. After centrifugation at 1,000 ×g for 20 minutes at 4 ℃, the supernatant was carefully collected and stored at −80 °C. CTRP4 (MM-1640H2), IL-6 (MM-0049H1), and TNF-α (MM-0122H1) ELISA kits were purchased from Meimian Biotechnology (Yancheng, Jiangsu, China). Serum samples (100 μL) were analyzed three times.

The individual reference and patient samples were added to the microtiter plate. The standard concentration was determined as: 4,000, 2,000, 1,000, 500, 250, and 125 pg/mL. One hundred μL of serum was added to the appropriate wells according to the manufacturer instructions. One hundred μL of the enzyme conjugate was added to the appropriate wells and incubated for 1h at RT. Each plate was washed 4 times, and 50 μL of each substrate (liquid A and B) was added to each well. Next, the substrates were incubated for 15 minutes at 37 °C. Fifty μL of the stopping solution was added to each well, and the plate was tapped gently to mix thoroughly. The optical density (O.D.) was read at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Immunohistochemistry (IHC) staining

Human brain tissue paraffin sections were obtained as described previously. We stained 4-μm-thick sections with IHC. For IHC staining, primary antibody-CTRP4 from Proteintech (Rabbit Polyclonal, 1:100, Catalog number: 14023-1-AP, USA) was used, and the Polymer HRP Detection System was bought from Bioss (Rabbit, PV-0023). The DAB Immunohistochemistry Color Development Kit was purchased from BBI (E670033-0100). Each presented immunohistochemistry was repeated at least three times. Images were acquired with a microscope (magnify, ×400).

Clinical data collection

Related clinical information was collected, including gender, age, clinical symptoms, CTRP4 concentration, cerebrospinal fluid (CSF) protein, CSF leucocytes, and MRI. HSE patients were graded with MMSE clinical scores on admission. MMSE can comprehensively, accurately, and quickly reflect the mental state of the subjects and the degree of their cognitive impairment. MMSE was a general guideline for the progression of HSE progress. Patients enrolled had a similar educational background. Then, we analyzed the correlation between this data and the CTRP4 expression.

Statistical analysis

Data were presented as the mean ± SD and analyzed with SPSS 17.0. One-way analysis of variance or the repeated measures were used to judge the statistical significance of the differences, and a Spearman's rank correlation coefficient for bivariate correlations between CTRP4 and inflammatory markers was completed. Values of *P<0.05, **P<0.01, and ***P<0.001 were considered statistically significant.

Results

Bioinformatics analysis

CTRP4 showed a higher expression in HSE than in normal brain tissues in the mice (*Figure 1*). The expression of CTRP4 in HSV-1 infected brain (mean intensity =27,754.7) was higher than that of control samples (mean intensity =9,473.0), and the fold change was 2.93 (P=0.019). CTRP4 showed a comparable level of expression between BM (mean intensity =783.26) and normal brain (mean intensity =876.71) tissue in mice (P=0.7143). There was no significant difference between BM and the normal group. Similarly, CTRP4 showed a comparable level of expression between TBM (mean intensity =2,662) and normal brain (mean intensity =6,339) tissue. In human tissues (P=0.0946), there was no significant difference between the TBM and normal group.

ELISA

Expression of CTRP4 was higher in HSE serum than in the normal serum (P<0.001), the BM serum, and the TBM serum. The average CTRP4 value in the normal serum was

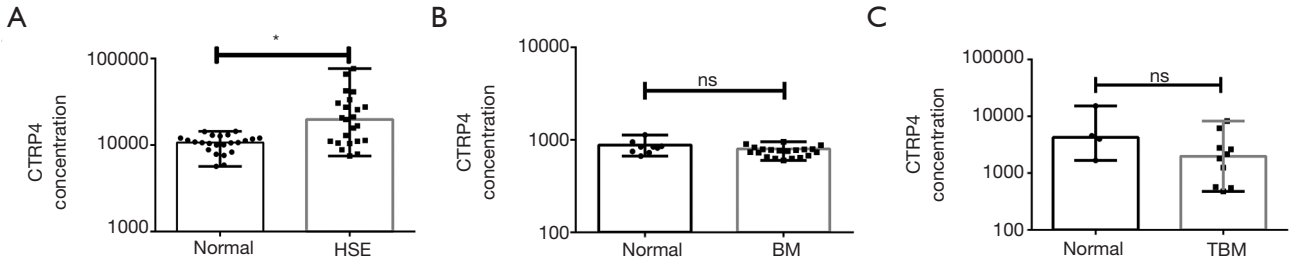

Figure 1 CTRP4 expression level analyzed by bioinformatics. (A) CTRP4 expression level analyzed between normal and HSE, *, P<0.05; (B) CTRP4 expression level analyzed between normal and BM; no significance between two groups; (C) CTRP4 expression level analyzed between normal and TBM; no significance between two groups. ns, no significance; BM, bacterial meningitis; CTRP4, C1Q/TNF-related protein 4; HSE, herpes simplex encephalitis.

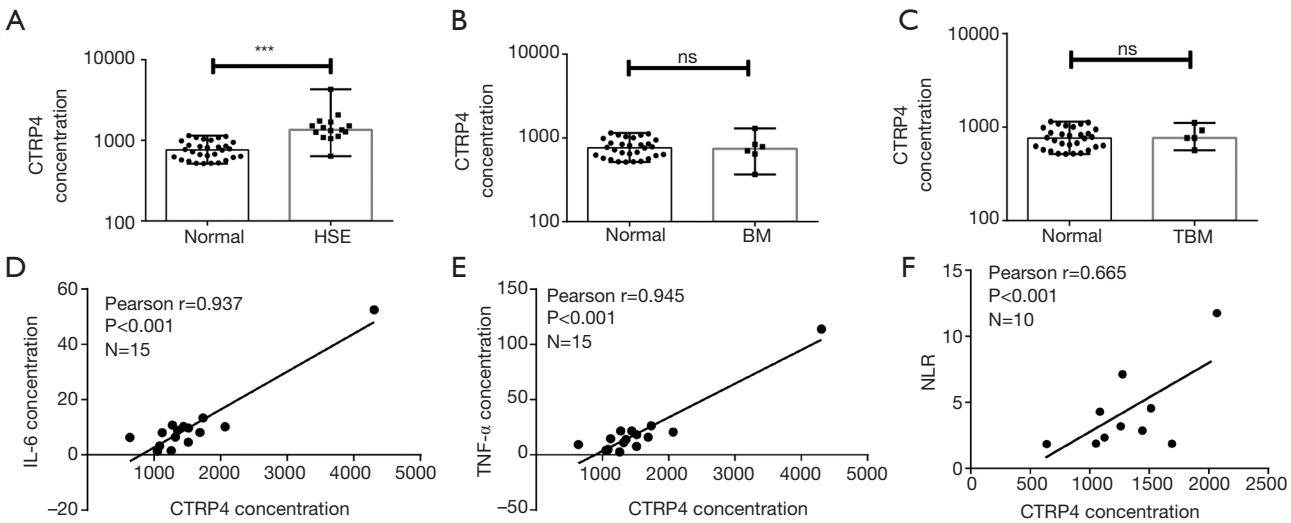

Figure 2 Detection and correlation analysis of CTRP4 concentration and pro-inflammatory cytokines in HSE patients' serum samples. (A) Comparison of sCTRP4 concentration between the normal group and HSE group, ***P<0.001; (B) comparison of sCTRP4 concentration between normal group and BM group; (C) comparison of sCTRP4 concentration between the normal group and TBM group; (D) the correlation analysis between SIL-6 and sCTRP4, r=0.937; (E) the correlation analysis between TNF-α and sCTRP4, r=0.945; (F) the correlation analysis between NLR and sCTRP4, r=0.665. BM, bacterial meningitis; CTRP4, C1Q/TNF-related protein 4; HSE, herpes simplex encephalitis; TBM, tuberculous meningitis; NLR, neutrophil lymphocyte ratio; ns, no significance.

775.804, 1,559.387 in the HSE serum, 826.2 in the TBM serum, and 772.5 in the BM serum. In the HSE serum, the average IL-6 value was 10.3265, and the average TNF-α value was 20.386. The correlation coefficient of CTRP4 and IL-6 was 0.937, and the correlation coefficient of CTRP4 and TNF-α was 0.945 (*Figure 2*).

Validation data in the patients' cohort

Expression of CTRP4 was higher in the HSE brain sample than the normal brain sample, BM brain sample, and TBM brain sample in humans. CTRP4 showed strong positive staining in HSE brain, but weak staining in the normal brain, TBM brain, and BM brain (*Figure 3*).

Correlation of CTRP4 expression with progression of HSE

The patient MRI showed long T1, and T2 signal accompanied high CTRP4 and serious syndrome before treatment. An MRI of the same patient showed shrinking lesions that were accompanied by lower CTRP4 with syndromes being relieved after treatment.

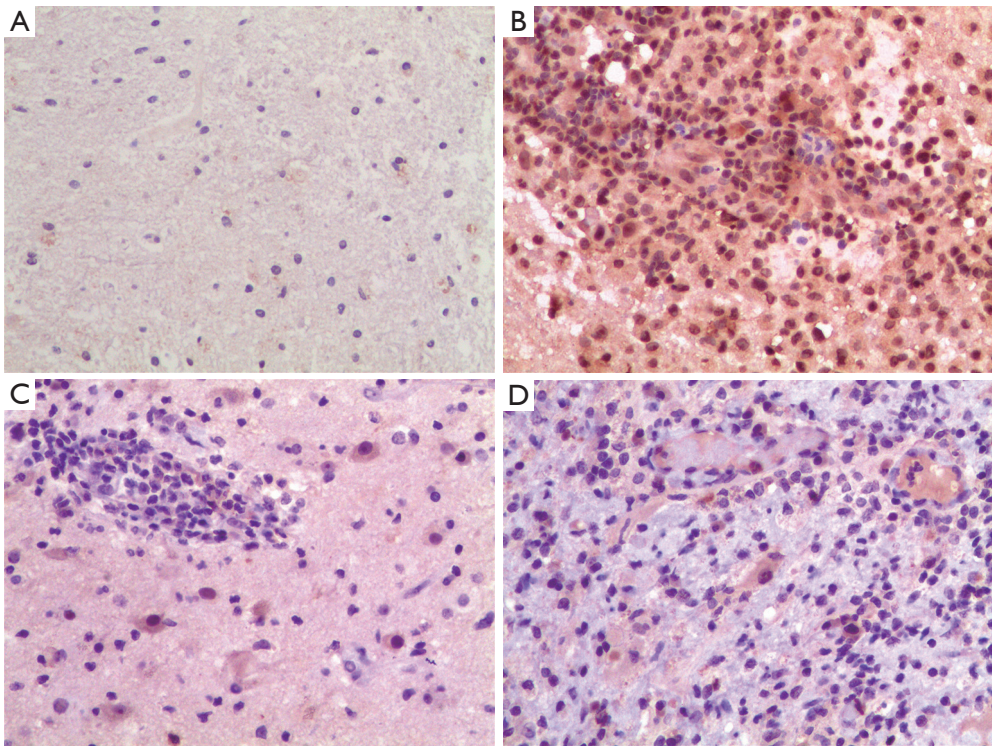

Figure 3 Normal brain, HSE, BE, and TBM tissues were immunostained by a polyclonal antibody against CTRP4 protein (EnVision, ×400). (A) Normal brain tissue was stained weakly with CTRP4 in individual glial cells; (B) HSV brain tissue was stained weakly with CTRP4 in most glial cells and lymphocytes; (C) BM brain tissue was stained weakly with CTRP4 in scattered glial cells and lymphocytes; (D) TBM brain tissue was stained weakly with CTRP4 in scattered glial cells and lymphocytes. BM, bacterial meningitis; CTRP4, C1Q/TNF-related protein 4; HSE, herpes simplex encephalitis; TBM, tuberculous meningitis.

We analyzed the detailed clinical information including the MMSE table from the 14 HSE patients (*Table 1*), and significant correlations were found between CTRP4 concentration and MMSE scores (*Figure 4*).

Discussion

HSE is a life-threatening condition with high mortality as well as a significant morbidity rate in survivors. Histopathologically, HSV causes hemorrhagic necrosis, accompanied by neuronal intranuclear inclusion bodies (INIBs) (4,16). Although the pathogenesis of HSE remains unclear, viral-infection-induced excessive inflammation is believed to play a crucial role. IL-6 and TNF-α are the most common pro-inflammatory cytokines (17,18). The IL-6/STAT3 axis plays an important role in the pathogenesis of HSE. The study showed that IL-6 combined with the STAT3 signaling cascades played an important role in the inflammatory reaction. During the early stage of an HSV-1 infection, STAT3 had protective effects by mediating cellular responses to multiple cytokines, governing gene expression and regulating the development and activation of immune cells (19).

In HSE animal models, studies showed that HSE inhibitors could specifically suppress HSV-induced activation of inflammation factors (20). IL-6 protects from HSE likely through its downstream STAT3 cascade (17). In mice models, STAT3 took part in HSE progression. STAT3 KO mice were more susceptible to an HSV-1 infection compared to the wild type mice. STAT3 pathway modulates HSV-1 reactivation and governs its infections (18,21-23). Phosphorylated STAT3 was suggested to be correlative with protective effects in the analysis of many transcription factors.

CTRP family is suggested to be involved in inflammation (16,20). The structure of CTRP4 includes two globular domains, whereas the other CTRPs contain only one globular domain. CTRP4 is produced as a classical

Table 1 HSE patients' clinical data and related examinations on admission

Sample number	Gender	Age, years	Clinical symptoms	MMSE	CTRP4	CSF leucocytes (μL)	CSF protein	MRI
Sample 1	Male	15	Headache, fever, seizures, consciousness loss	21	1,442.782	31	52 mg/dL	High signal on T2 in the left temporal and occipital lobes with edema, uneven enhancement in the left lateral temporal region
Sample 2	Male	60	Headache, seizures	23	1,350.276	22	53 mg/dL	High signal on T2 in the right temporal lobe
Sample 3	Female	46	Headache, fever, abnormal behavior, seizures	25	1,085.345	66	136 mg/dL	High signal on T2 in bilateral frontal, temporal lobes and right occipital lobe
Sample 4	Male	27	Fever, abnormal behavior, confusion, or disorientation	18	1,260.343	68	66 μL	High signal on T2 in bilateral frontal, temporal and insular lobes
Sample 5	Male	27	Headache, fever, consciousness loss, abnormal behavior	16	2,068.739	357	74.82 mg/dL	High signal on T2 in bilateral temporal lobes
Sample 7	Female	35	Fever, seizures, consciousness loss	20	1,514.762	28	27.61 mg/dL	High signal on T2 in bilateral hippocampus, insular temporal lobes
Sample 8	Female	33	Headache, seizures, hypomnesia	22	1,124.168	29	23.07 mg/dL	High signal on T2 in bilateral hippocampus insular lobes and right frontal, temporal lobes
Sample 9	Male	17	Fever, seizures, abnormal behavior, confusion or disorientation	0	4,306.301	278	102.68 mg/dL	High signal on T1 in the left temporal lobes with hemorrhagic transformation
Sample 10	Male	36	Fever, headache, abnormal behavior	19	1,275.969	630	76 mg/dL	High signal on T2 in bilateral temporal lobes
Sample 11	Female	31	Seizures, abnormal behavior, consciousness loss,	16	1,353.098	35	30.04 mg/dL	High signal on T2 in bilateral temporal lobes
Sample 12	Female	52	Fever, headache, seizures, consciousness loss	22	1,051.686	112	53.38 mg/d	High signal on T2 in the right temporal lobe
Sample 13	Male	27	Fever, headache, consciousness loss	21	1,517.442	35	136.1 mg/d	High signal on T2 in bilateral temporal lobes
Sample 14	Male	32	Fever, headache, seizures, abnormal behavior	22	1,738.786	16	36.2 mg/d	High signal on T2 in bilateral temporal lobes and right insular lobe

MMSE, mini-mental state examination; CTRP4, C1Q/TNF-related protein 4; CSF, cerebrospinal fluid.

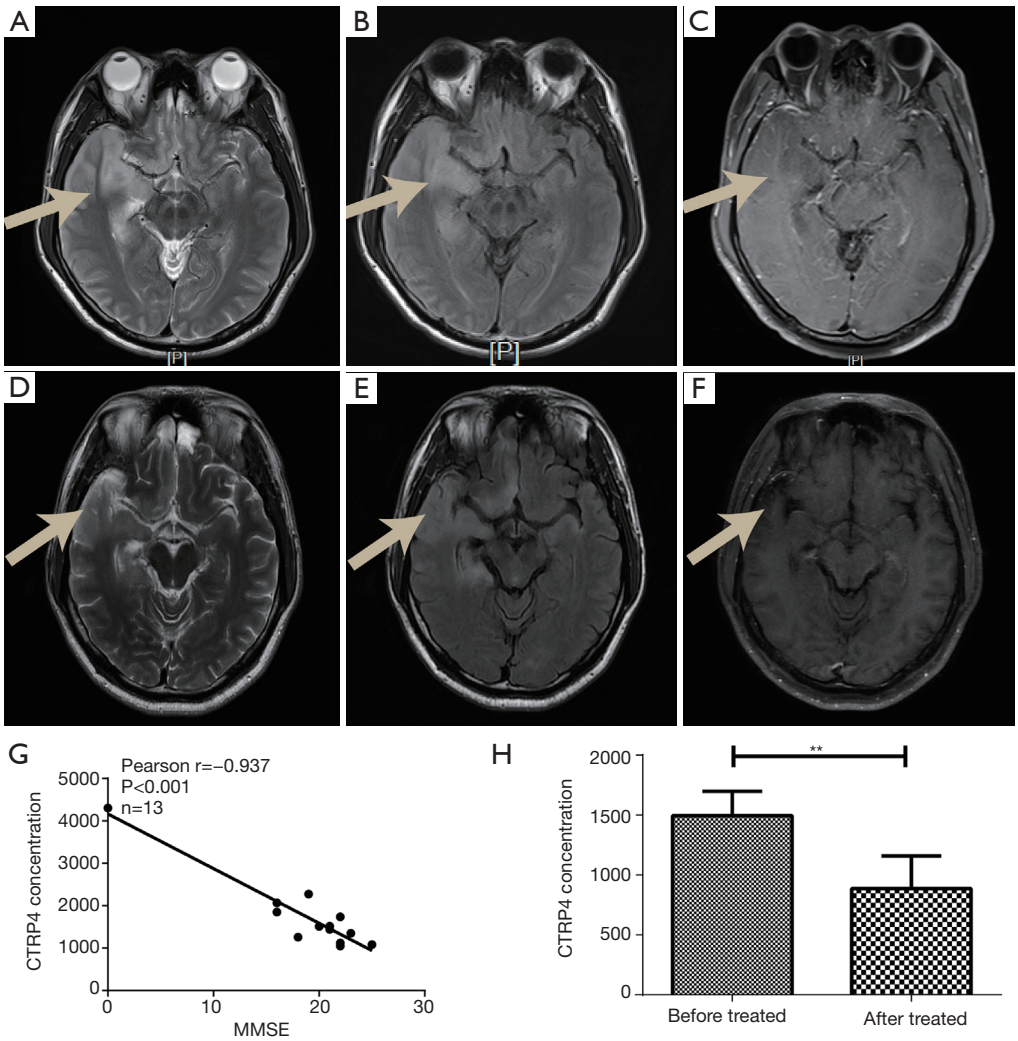

Figure 4 The correlation between CTRP4 concentration and patients' clinical parameters. (A) Lesion in T2 before treatment (arrow); (B) lesion in FLAIR before treatment (arrow); (C) lesion in T1 enhanced sequence before treatment (arrow); (D) lesion in T2 after treatment (arrow); (E) lesion in FLAIR after treatment (arrow); (F) lesion in T1 enhanced sequence after treatment (arrow); (G) the correlation analyzed between CTRP4 concentration and patients' clinical MMSE score, r=−0.937; (H) CTRP4 concentration analysis between pre- and post-treatment, **P<0.01. BM, bacterial meningitis; CTRP4, C1Q/TNF-related protein 4; HSE, herpes simplex encephalitis; TBM, tuberculous meningitis.

secreted protein. Research showed that the overexpression of CTRP4 could increase IL-6 expression and activation of STAT3 in HepG2 cells, indicating that CTRP4 and IL-6 are correlatively upregulated during inflammation (15). In dextran sulfate sodium (DSS)-induced acute colitis model, the expression of endogenous CTRP4 was greatly up-regulated, and phosphorylation of STAT3 was downregulated in rhCTRP4-treated mice. Expression of IL-6 and TNF-α and phosphorylation of STAT3 were decreased in the rhCTRP4-treated group when compared to the control group. CTRP4 can trigger the pathway of both NF-kB and IL6/ STAT3 (13,15,24). In coronary heart disease, the serum CTRP4 concentration is elevated correlating with inflammatory factors such as TNF-α and IL-6. CTRP4 was elevated in HSE serum compared to TBM, BM, and normal serum. In HSE, CTRP4 concentration showed similar kinetics with other inflammatory factors such as IL-6 and TNF-α. The high expression of CTRP4 correlated with

the patient's symptoms, MRI imaging, and MMSE clinical score. It might be that CTRP4 takes part in the regulation of innate immune response in the human brain.

Elevated levels of neutrophil lymphocyte ratio (NLR) were found to be associated with a poor survival rate of patients who had undergone a coronary artery bypass graft (25). Many cancer survival studies have suggested that NLR is a significant predictor of overall and disease-specific survival of patients (26,27). Systemic inflammation measured by NLR has a significant association with prevalent chronic conditions (28). We found a correlation between NLR and CTRP4 level (*Figure 2F*). The neutrophil-lymphocyte ratio could be an important measure of systemic inflammation as it is cost effective, readily available, and can be calculated easily. The potential value of CTRP4 in predicting the outcome of HSE needs further investigation.

Conclusions

In summary, CTRP4 is closely correlated with HSE progression and may serve as an indicator of the disease's severity. The mechanism of CTRP4 in HSE may be related to IL-6-mediated inflammatory responses including STAT3 signaling. Moreover, the mechanism of CTRP4 remains to be further studied. Nevertheless, our study highlights the potential significance of CTRP4 in the progression of HSE and provides a novel intervention perspective in the treatment and prognosis of HSE.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: This study was approved by the Ethics Committee of Beijing Tiantan Hospital, Beijing Ditan Hospital, and Capital Medical University (NO. KYSQ 2019-011-01) and written informed consent was obtained from all patients.

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