Elevated cerebrospinal fluid endothelin 1 associated with neurogenic pulmonary edema in children with enterovirus 71 encephalitis

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A R T I C L E   I N F O

S U M M A R Y

Objectives: Neurogenic pulmonary edema (NPE) is a fatal complication in children with enterovirus 71 (EV71) encephalitis. Endothelin 1 (ET-1), a potent vasoconstrictor, can induce pulmonary edema in rats via intrathecal injections. Thus, it was hypothesized that ET-1 in the central nervous system may correlate with NPE in children with EV71 encephalitis.
Methods: Clinical data and ET-1 in the cerebrospinal fluid (CSF) were compared between three groups: (1) EV71 encephalitis with NPE; (2) EV71 encephalitis without NPE; and (3) non-EV71 aseptic meningitis. ET-1 immunostaining was performed on the brainstem of autopsy patients.
Results: The EV71 with NPE group showed significantly increased CSF levels of ET-1 compared to the EV71 without NPE and the non-EV71 aseptic meningitis groups (both $p < 0.01$). The optimum cut-off point of ET-1 to predict NPE in EV71 patients, based on the receiver operating characteristic curve, was 0.5 pg/ml (sensitivity 83%, specificity 100%). Immunostaining in the brainstem showed increased ET-1 expression, mainly in the oligodendrocytes, in EV71 with NPE patients compared with control patients.
Conclusion: ET-1 in the central nervous system may play a role in the development of NPE in children with EV71 infection and could be used as a biomarker or therapeutic target for NPE in EV71 encephalitis.
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1. Introduction

Enterovirus 71 (EV71) belongs to the human *Enterovirus A* species within the family *Picornaviridae*. EV71 is a frequent cause of hand, foot and mouth disease, which can be complicated by severe neurological involvement in children, especially those younger than 5 years of age.[1] Most of the fatal cases initially present with minor neurological symptoms, rapidly become complicated with autonomic neurological dysfunction and neurogenic pulmonary edema (NPE), and die of cardiopulmonary failure soon after admission. The mortality from this condition ranges from 80% to 90%.[1] The presence of NPE is usually the main cause of fatal EV71 during epidemics, but its pathogenesis is not completely understood. Brain magnetic resonance imaging (MRI) studies have shown a propensity of EV71 infection for the tegmentum of the pons and medulla oblongata. Post-mortem studies have demonstrated that patients who died from EV71-induced NPE had acute inflammation and EV71 antigens within neurons in the brainstem.[2,3] Thus, brainstem encephalitis is speculated to be the cause of acute fatality with NPE after EV71 infection.[4]

Endothelin 1 (ET-1), a 21-amino acid peptide, is a potent vasoconstrictor.[5] Because its mRNA and receptor binding sites are expressed in several regions of the brain, ET-1 can also work as a neurotransmitter to regulate neurological functions.[6,7] Several animal studies have shown that intrathecal injection of ET-1 elicits a transient increase in arterial blood pressure, heart rate, and

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sympathetic nerve activity.[8] Intrathecal injection of ET-1 also enhances pulmonary vascular permeability and causes pulmonary edema.[7] It was concluded that pulmonary edema was due to intense pulmonary vasoconstriction mediated by α-adrenoceptors following the release of catecholamines in response to the activation of endothelin receptors in the central nervous system.[7] Thus, ET-1 plays a role in inducing NPE.

It was hypothesized that the occurrence of NPE in children with EV71 infection is correlated with an increase in ET-1 in the brainstem. Cerebrospinal fluid (CSF) samples and autopsy brain specimens from patients with EV71 infection were used to investigate this issue.

2. Materials and methods

2.1. Patients

The cases of 16 patients with EV71 encephalitis who had been admitted to the study hospital during the EV71 epidemic from 1998 to 2000 in southern Taiwan were reviewed. The inclusion criteria for the patients with EV71 encephalitis were the following: (1) defined EV71 infection by viral isolation, (2) presence of neurological manifestations including headache, altered consciousness, myoclonic jerks, ataxia, tremor, acute flaccid paralysis, bulbar palsy, or NPE, and (3) CSF leukocyte count >10 × 10^6 cells/l.[1] NPE was defined as the occurrence of respiratory distress, tachycardia, tachypnea, and copious frothy sputum, with chest radiological findings of bilateral pulmonary infiltrates without cardiomegaly. The 16 patients who had EV71 encephalitis were further stratified into two groups: EV71 encephalitis without NPE ($n = 10$) and EV71 encephalitis with NPE ($n = 6$).

An additional control group comprised nine patients with non-EV71 aseptic meningitis who were admitted during the same period. The diagnosis of non-EV71 aseptic meningitis was based on the following criteria: (1) CSF leukocyte count >10 × 10^6 cells/l with a predominance of mononuclear cells, (2) negative bacterial studies, including CSF and blood cultures, and (3) self-limited clinical course without clinical evidence of encephalopathy, such as seizures, disturbances of consciousness, or focal neurological signs.

All of the medical charts, laboratory results, and MRI scans were reviewed retrospectively. The study was approved by the institutional review board of the National Cheng Kung University Hospital.

2.2. Virology studies

EV71 was confirmed by virus isolation as described previously.[1] Briefly, specimens were inoculated onto monolayers of A549, Vero, and green monkey kidney cells within 24 h and then incubated and evaluated for evidence of a viral cytopathic effect. Isolates were further typed using an immunofluorescence assay with EV71 monoclonal antibodies 3323 and 3324 (Chemicon International, Temecula, CA, USA). EV71 was identified by positive staining of both antibodies. These isolates were further confirmed by neutralization testing using polyclonal antiserum (American Type Culture Collection, Rockville, MD, USA).

2.3. Analysis of ET-1

Archived CSF specimens had been stored in cryogenic vials in −80 °C freezers; these specimens had been placed in the freezers within 4–6 h after collection. ET-1 concentrations were determined using the QuantiGlo Human ET-1 Chemiluminescent Immunoassay (R&D Systems, Minneapolis, MN, USA). According to the manufacturer, the sensitivity of the ET-1 assay is 0.102 pg/ml. In brief, a monoclonal antibody specific for ET-1

was pre-coated onto a microplate to bind ET-1 in standards and samples. After washing, an enzyme-linked monoclonal antibody specific for ET-1 was added to the wells. Following a wash, an enhanced luminol/hydrogen peroxide substrate solution was added and light was produced in proportion to the amount of ET-1 bound in the initial step. A microplate luminometer (Luminoskan Ascent; Thermo Scientific, Waltham, MA, USA) was used to measure the intensity of the light emitted. The standard curve was modified from the R&D Systems recommendations to include 0.04, 0.2, 1, and 5 pg/ml standards. Raw data were transferred to a computer in which a log concentration–log RLU (relative light unit) equation was defined and sample concentrations were calculated.

2.4. Immunohistochemistry and double-immunofluorescence staining

ET-1 immunohistochemistry was performed on selected blocks of nervous tissue from a patient who had died in the EV71 encephalitis with NPE group. To compare the differences in ET-1 expression in the brainstem, another age-matched patient who had died of severe neuroblastoma without brainstem metastasis was used as a control. Tissue sections (10 μm in thickness) were deparaffinized with xylene and graded ethanol solutions. The antigen was retrieved by microwaving for 10 min in sodium citrate buffer, pH 6.0. For immunohistochemistry, tissue sections were blocked with 2% normal goat serum and 0.1% Triton X-100, and probed with primary antibodies to ET-1 (1:200, Abcam) at 4 °C overnight, followed by a 2-h incubation with horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibodies at room temperature. Biotin-peroxidase signals were detected using 0.5 mg/ml 3,3'-diaminobenzidine/0.003% H_2O_2 as a substrate. For double-immunofluorescence staining, tissue sections were probed with primary antibodies anti-microtubular-associated protein 2 (MAP-2) (1:50, Cell Signaling), anti-CD68 (1:50, Santa Cruz), anti-Iba1 (1:500, Wako), anti-glial fibrillary acidic protein (GFAP) (1:500, Abcam), anti-Oligo2 (1:500, Abcam), or anti-ET-1 (1:200, Abcam) at 4 °C overnight after blocking. The sections were then incubated with Alexa Fluor 488 goat IgG and Alexa Fluor 594 goat IgG secondary antibodies (Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature after washing. Images were acquired on a Nikon E400 fluorescence microscope (Tokyo, Japan). Digitally captured images were analyzed using Imaging Software NIS-Elements (Nikon, Tokyo, Japan).

2.5. Statistical analysis

Significant differences between the study groups were assessed using a commercial software program (SPSS version 17.0; SPSS Inc., Chicago, IL, USA). One of the authors (CHL), who was blinded to the study design, performed all the statistical analyses. Continuous data are presented as the mean ± standard deviation (SD) and were tested by non-parametric Mann–Whitney U-test. Categorical variables were tested by Chi-square test or Fisher's exact test. A receiver operating characteristic (ROC) curve was constructed to assess the sensitivity and specificity of ET-1. A p-value of <0.05 was considered statistically significant, and all probabilities were two-tailed.

3. Results

3.1. Study patients

The demographic information and clinical manifestations of the three groups are shown in Table 1. Although the EV71 encephalitis with NPE group was younger at disease onset, the age at onset was comparable between the three groups. On admission, the EV71

Table 1
Demographic and clinical findings of the three groups of patients[a]

	EV71 encephalitis with NPE (n = 6)	EV71 encephalitis without NPE (n = 10)	Non-EV71 aseptic meningitis (n = 9)	p-Value[b]	p-Value[c]
Age, months	24.2 ± 17.2	39.2 ± 32.2	53 ± 75.4	NS	NS
Male/female, n/n	4/2	6/4	5/4	NS	NS
Vital signs at admission					
Body temperature, °C	37.7 ± 0.6	37.8 ± 0.5	37.9 ± 1.3	NS	NS
Heart rate, /min	163 ± 21	131 ± 15	110 ± 26	0.01	0.006
Arterial BP, mmHg					
Systolic	132 ± 8	114 ± 15	112 ± 10	0.037	0.027
Diastolic	77 ± 12	72 ± 18	84 ± 42	NS	NS
Respiratory rate, /min	42 ± 14	28 ± 8	33 ± 13	0.019	NS
Neurological symptom/signs, n (%)					
Altered consciousness	5 (83.3%)	0 (0%)	0 (0%)	0.001	0.002
Limb paralysis	3 (50%)	0 (0%)	0 (0%)	0.036	0.044
Myoclonus	3 (50%)	10 (100%)	0 (0%)	0.036	0.044
Ataxia/tremor	2 (33.3%)	1 (10%)	0 (0%)	NS	NS
Bulbar involvement	2 (33.3%)	0 (0%)	0 (0%)	NS	NS
Seizure	2 (33.3%)	0 (0%)	0 (0%)	NS	NS
Headache	1 (16.7%)	2 (20%)	4 (44.4%)	NS	NS
Admission course					
Dopamine, n (%)/days	4 (66.7%)/1.8 ± 0.5	0 (0%)/0	0 (0%)/0	0.008	0.008
Dobutamine, n (%)/days	4 (66.7%)/2.8 ± 0.9	0 (0%)/0	0 (0%)/0	0.008	0.008
Intubation, n (%)/days	6 (100%)/2.2 ± 1.2	0 (0%)/0	0 (0%)/0	<0.001	<0.001
Outcome, n (%)					
Death	1 (16.7%)	0 (0%)	0 (0%)	NS	NS
Neurological sequelae	3/5 (60%)	0 (0%)	0 (0%)	0.022	0.027

BP, blood pressure; EV71, enterovirus 71; NPE, neurogenic pulmonary edema; NS, not significant.
[a] Results are presented as the mean ± standard deviation, unless stated otherwise.
[b] p-Values for comparisons between the EV71 with NPE and EV71 without NPE groups.
[c] p-Values for comparisons between the EV71 with NPE and non-EV71 aseptic meningitis groups.

with NPE group had significantly higher heart rates and systolic arterial blood pressures than the EV71 without NPE group and the non-EV71 aseptic meningitis group (all $p < 0.05$). The most common neurological manifestation was altered consciousness in EV71 with NPE patients, myoclonus in patients with EV71 without NPE, and headaches in patients with non-EV71 aseptic meningitis.

3.2. Clinical course and outcome

All of the patients with EV71 and NPE had respiratory failure that required intubation for oxygen and respiratory support, and most were also treated with inotropic agents such as dopamine/dobutamine for hypotension or shock. None of the patients with EV71 without NPE or the non-EV71 patients with aseptic meningitis had respiratory failure or hypotension (Table 1). In the EV71 with NPE group, one patient died due to cardiopulmonary failure and three patients had persistent limb paralysis on follow-up. None of the patients in the EV71 without NPE group or in the non-EV71 aseptic meningitis group had abnormal neurological outcomes.

3.3. CSF ET-1 levels

Total white blood cell counts in the CSF of the three groups were similar. A slight predominance of CSF mononuclear cells in the patients with non-EV71 aseptic meningitis compared with patients in the EV71 with and without NPE groups was noted (Table 2). The patients in the EV71 with NPE group had significantly higher CSF levels of ET-1 (mean ± SD, 0.56 ± 0.09 pg/ml) than the patients in the EV71 without NPE group (0.32 ± 0.14 pg/ml; $p < 0.002$) and the patients in the non-EV71 aseptic meningitis group (0.22 ± 0.14 pg/ml; $p < 0.004$) (Figure 1A). CSF levels of ET-1 were similar in the EV71 without NPE and the non-EV71 with aseptic meningitis groups. The area under the ROC curve of ET-1 for the diagnosis of NPE in EV71-infected patients was 0.967 (Figure 1B). The best cut-off point was 0.5 pg/ml, with a sensitivity of 83% (95% confidence interval 36.5% to 99.1%) and specificity of 100% (95% confidence interval 65.5% to 100%).

3.4. Brainstem lesions and ET-1 expression

MRI was available for four patients in the EV71 with NPE group. Three patients showed high T2 signal lesions in the dorsal medulla

Table 2
CSF data[a]

	EV71 encephalitis with NPE (n = 6)	EV71 encephalitis without NPE (n = 10)	Non-EV71 aseptic meningitis (n = 9)	p-Value[b]	p-Value[c]
WBC, ×10⁶ cells/l	126 ± 127	103 ± 175	68 ± 66	NS	NS
Segmented neutrophils (%)	34 ± 40	70 ± 29	7 ± 10	NS	NS
Lymphocytes (%)	36 ± 42	18 ± 18	69 ± 29	NS	0.05
Lactate, mM/l	3.2 ± 3.9	1.4 ± 0.5	1.4 ± 0.3	NS	NS
Protein, mg/dl	57 ± 27	40 ± 21	59 ± 27	NS	NS
Glucose, mg/dl	72 ± 8	64 ± 10	58 ± 13	NS	NS

CSF, cerebrospinal fluid; EV71, enterovirus 71; NPE, neurogenic pulmonary edema; NS, not significant; WBC, white blood cell count.
[a] Results are presented as the mean ± standard deviation.
[b] p-Values for comparisons between EV71 with NPE and EV71 without NPE groups.
[c] p-Values for comparisons between EV71 with NPE and non-EV71 aseptic meningitis groups.
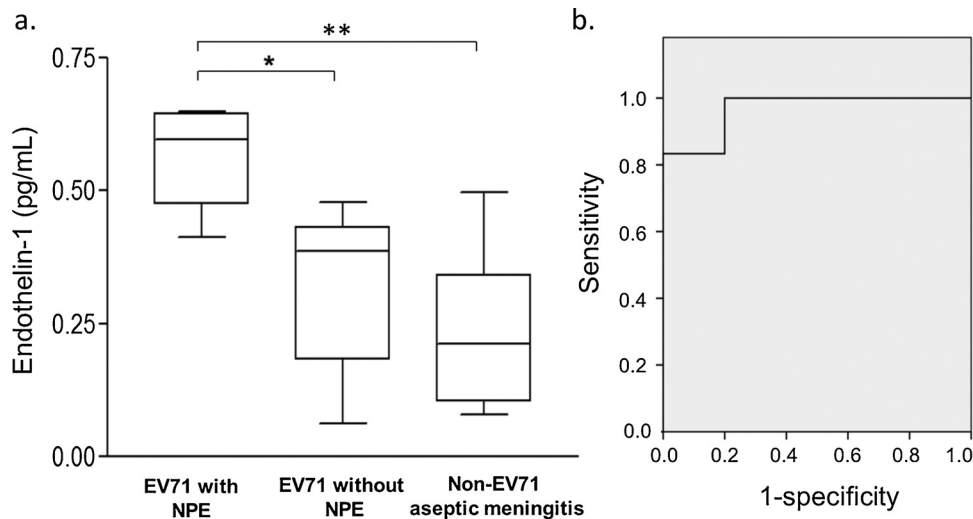
Figure 1. ET-1 values in the CSF. (A) The EV71 encephalitis with NPE group had significantly higher CSF levels of ET-1 than the EV71 encephalitis without NPE group and the non-EV71 with aseptic meningitis group; *p-value = 0.005, **p-value = 0.004. (B) ROC curve of CSF ET-1 levels for the diagnosis of NPE caused by EV71 infection. (Abbreviations: ET-1, endothelin 1; CSF, cerebrospinal fluid; EV71, enterovirus 71; NPE, neurogenic pulmonary edema; ROC, receiver operating characteristic.)

oblongata, with or without extension to the pontine tegmentum, and two also had a longitudinal high T2 signal lesion at the spinal cord. The MRI of the patient who died of EV71 infection with NPE showed T2 hyperintensity lesions at the pontine tegmentum and dorsal medulla oblongata (Figure 2A). ET-1 immunohistochemistry in the medulla oblongata of this patient showed increased cellular ET-1 expression compared with a patient with a high-grade neuroblastoma without brain metastasis (Figure 2B). Immuno-fluorescence showed that the upregulated expression of ET-1 did not co-express in MAP-2-positive neurons, CD68-positive macrophages, Ib1-positive microglia, or GFAP-positive astrocytes. Instead, ET-1 was mainly co-localized with the Oligo2-positive oligodendrocytes (Figure 3).

4. Discussion

Since its first discovery in 1969, EV71 has caused several major epidemics of hand, foot and mouth disease complicated with severe neurological sequelae throughout the world.[4,9] In the 1998 Taiwan outbreak, 1.5 million people were infected, more than 400 children were admitted with neurological complications, and up to 78 children died of complications, such as NPE and cardiopulmonary failure.[10] Even though several pathogenic mechanisms of NPE after EV71 infection have been proposed, the exact mechanism remains unclear.[4] The present study provided clinical evidence that EV71-infected patients with NPE had increased ET-1 in the CSF compared to EV71-infected patients without NPE and non-EV71 patients with aseptic meningitis. Immunohistochemistry also confirmed increased ET-1 expression in the medulla oblongata of a patient with fatal EV71 infection and NPE. Interestingly, ET-1 was expressed mainly in oligodendrocytes instead of neurons, macrophages, microglia, or astrocytes. These findings suggest that ET-1 produced by oligodendrocytes may play a role in the development of NPE in EV71-induced brainstem encephalitis.

NPE is characterized by dyspnea, bilateral basal pulmonary crackles, and the absence of cardiac failure.[11] NPE usually occurs within minutes to hours after a central neurological insult that causes damage to potential NPE trigger zones in the brainstem, especially the vasomotor centers in the medulla oblongata and hypothalamus, and leads to massive sympathetic discharges.[12,13] The significant alterations in the autonomic nervous system in turn increase lung capillary permeability or pulmonary vascular hydrostatic pressures, and cause pulmonary edema.[14,15] Animal experiments have shown that damage to the medulla oblongata can produce profound transient pulmonary and systemic hypertension and pulmonary edema.[16–18] These responses can be abolished by α-adrenergic blockade (with phentolamine) or spinal cord transection at the C7 level.[13,17] Thus, NPE may be caused by damage to the medulla oblongata through activating sympathetic tone.

Clinically, most children with EV71 encephalitis with NPE have had preceding sympathetic symptoms, such as tachycardia, tachypnea, and hypertension, before the onset of clinical deterioration.[10,19] Manifestations of cardiopulmonary failure, including hypotension and pulmonary edema, follow rapidly within hours.[20] Some patients have died soon after the deterioration. Neuroimaging and neuropathological studies have confirmed EV71-induced damage to the brainstem, where EV71 antigens, virus-like particles, and inflammation were found.[1,2,19,21] These clinical manifestations and pathological findings suggest that EV71 causes inflammation of the NPE trigger zone in the brainstem, over-activates sympathetic tone, and results in the development of NPE.

ET-1 is known to be a potent vasoconstrictor.[5] Animal studies have shown that intracerebroventricular injection of ET-1 (10 pmol) increases arterial blood pressure, heart rate, and sympathetic nerve activity.[8,22] Intracisternal administration of a higher dose of ET-1 (20 pmol) has been shown to result in hypotension, bradycardia, and 100% mortality.[23] Moreover, intrathecal ET-1 (650 pmol) has been shown to enhance pulmonary vascular permeability and cause pulmonary edema.[7] These changes induced by increasing doses of central ET-1 injection are quite similar to the progression of clinical symptoms in patients with EV71-induced NPE. In the brainstem, endothelin binding sites have been found in the vasomotor centers, including the nucleus of the solitary tract and rostroventrolateral medulla, which participates in cardiovascular function.[24,25] Thus, endogenous ET-1 induced by EV71 in the brainstem may elicit sympathetic tone through stimulation of vasomotor centers, enhance pulmonary vascular permeability, and lead to NPE. This concept is supported by the findings of the present study in which EV71-infected patients with NPE had increased ET-1 levels in the CSF and cellular ET-1 expression in the medulla oblongata. Taken together, ET-1 may be involved in the induction of NPE during EV71 infection, and CSF ET-1 could be a sensitive biomarker of NPE in patients with EV71 infection.
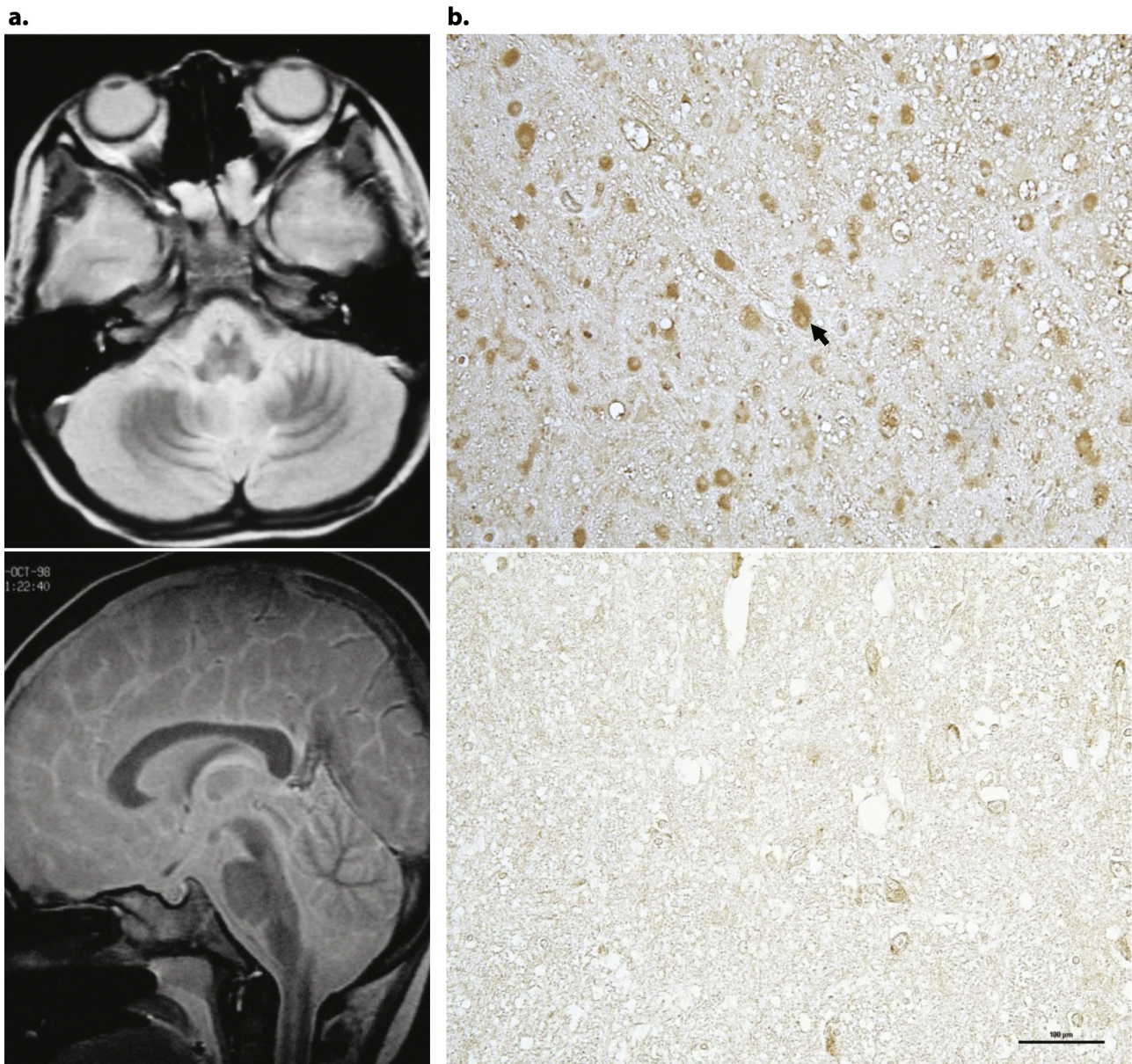
Figure 2. MRI and ET-1 immunohistochemistry in the brainstem of autopsy patients. (A) T2-weighted MRI (TR 3500 ms, TE 93 ms) showing hyperintense signals in the dorsal pons and medulla oblongata in a patient with EV71 encephalitis with NPE. (B) ET-1 immunohistochemistry revealed that a patient who died of EV71 encephalitis and NPE had increased intracellular ET-1 expression in the dorsal medulla oblongata (upper panel) compared to a control patient who died of neuroblastoma without brain metastasis (lower panel). ET-1-positive cells are brown in color and one is marked with an arrow (upper panel). Scale bar = 50 μm. (Abbreviations: MRI, magnetic resonance imaging; ET-1, endothelin 1; TR, repetition time; TE, echo time; EV71, enterovirus 71; NPE, neurogenic pulmonary edema.)

Oligodendrocytes have been demonstrated to synthesize immune-relevant proteins and respond specifically to immuno-logical exposures in various pathological situations.[26] For example, oligodendrocytes and their progenitor cells express interleukin (IL)-1β and its receptors during development, and IL-18 and its receptors in cases of white matter injury.[27,28] Thus, oligodendrocytes like microglia can be involved in the immune reactions to the central nervous system infection. In this study, it was found that the majority of ET-1-positive cells were Oligo2-positive oligodendrocytes. These data indicate that oligodendrocytes are the major cell origin that produces ET-1 in patients with NPE during EV71 infection. Although ET-1 can stimulate microglia lineage cells to express inflammatory cytokines, CSF cytokine changes have been shown not to be associated with EV71 clinical severity or outcomes.[29–31] Thus, ET-1 may function as a stimulator

to increase sympathetic activity instead of inducing inflammatory responses in the brain.[22]

In accordance with the present findings, it is hypothesized that EV-71 infection triggers oligodendrocytes to produce ET-1, which increases sympathetic activity initially through stimulation of vasomotor centers in the brainstem. The increased amount of ET-1 results in hypotension, bradycardia, and pulmonary edema. Further prospective studies involving a large population with EV71 infection are needed in order to validate these findings. The correlated ET-1 expression in serum or respiratory secretions could be investigated as well. There are two US Food and Drug Administration (FDA)-approved ET-1 antagonists – bosentan and ambrisentan – which have been used clinically in pulmonary artery hypertension patients. If a causal relationship between ET-1 and NPE pathogenesis is established in further studies, ET-1
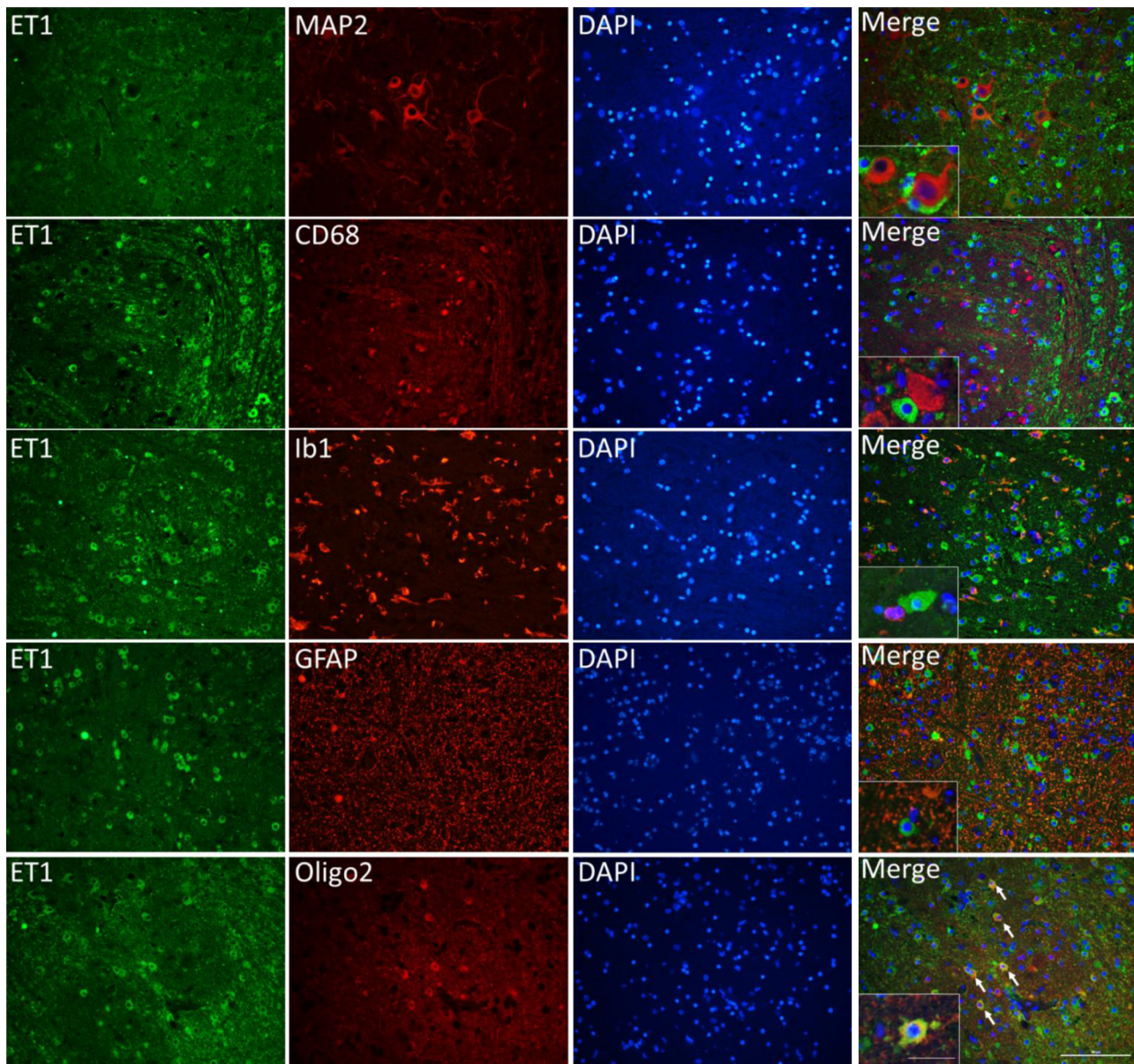
Figure 3. Immunofluorescence staining in the patient with fatal EV71 infection and NPE. Immunofluorescence staining in the patient with fatal EV71 infection and NPE showed that ET-1 was not expressed in neurons (MAP-2), macrophages (CD68), microglia (Ib1), or astrocytes (GFAP). Most ET-1 was co-localized with oligodendrocytes (Oligo2). Scale bar = 100 μm. High magnification images are shown in the inserts (scale bar = 50 μm). (Abbreviations: EV71, enterovirus 71; NPE, neurogenic pulmonary edema; ET-1, endothelin 1.)

antagonists might be used to rescue EV71-infected patients complicated with NPE.

This study provides the first evidence that CSF ET-1 is increased in patients with EV71-induced NPE. Because NPE is the leading fatal complication in children infected by EV71, early detection of NPE occurrence is critical. ET-1 could also be a potential therapeutic target for preventing or treating NPE in EV71 encephalitis.

Acknowledgements

Conflict of interest: The authors have no conflicts of interest to disclose.

References

1. Huang CC, Liu CC, Chang YC, Chen CY, Wang ST, Yen TF. Neurologic complication sin children with enterovirus 71 infection. *N Engl J Med* 1999;**341**:936–42.
2. Shieh WJ, Jung SM, Hsueh C, Kuo TT, Mounts A, Parashar U, et al. Pathologic studies of fatal cases in outbreak of hand, foot, and mouth disease, Taiwan. *Emerg Infect Dis* 2001;**7**:146–8.
3. Wong KT, Chua KB, Lam SK. Immunohistochemical detection of infected neurons as a rapid diagnosis of enterovirus 71 encephalomyelitis. *Ann Neurol* 1999;**45**: 271–2.
4. Solomon T, Lewthwaite P, Perera D, Cardosa MJ, McMinn P, Ooi MH. Virology, epidemiology, pathogenesis, and control of enterovirus 71. *Lancet Infect Dis* 2010;**10**:778–90.
5. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 1988;**332**:411–5.
6. Naidoo V, Mahabeer R, Raidoo DM. Cellular distribution of endothelin-1 mRNA in human brain by in situ RT-PCR. *Metab Brain Dis* 2001;**16**:207–18.

7. Poulat P, Couture R. Increased pulmonary vascular permeability and oedema induced by intrathecally injected endothelins in rats. *Eur J Pharmacol* 1998;**344**: 251–9.
8. Kuwaki T, Kurihara H, Cao WH, Kurihara Y, Unekawa M, Yazaki Y, et al. Physiological role of brain endothelin in the central autonomic control: from neuron to knockout mouse. *Prog Neurobiol* 1997;**51**:545–9.
9. Weng KF, Chen LL, Huang PN, Shih SR. Neural pathogenesis of enterovirus 71 infection. *Microbes Infect* 2010;**12**:505–10.
10. Chang LY, Lin TY, Hsu KH, Huang YC, Lin KL, Hsueh C, et al. Clinical features and risk factors of pulmonary oedema after enterovirus-71-related hand, foot and mouth disease. *Lancet* 1999;**354**:1682–6.
11. Sedy J, Likavcanova K, Urdzikova L, Zicha J, Kunes J, Hejcl A, et al. Low degree of anesthesia increases the risk of neurogenic pulmonary edema development. *Med Hypotheses* 2008;**70**:308–13.
12. Baumann A, Audibert G, McDonnell J, Mertes PM. Neurogenic pulmonary edema. *Acta Anaesthesiol Scand* 2007;**51**:447–55.
13. Malik AB. Mechanisms of neurogenic pulmonary edema. *Circ Res* 1985;**57**: 1–18.
14. Sedy J, Zicha J, Kunes J, Jendelova P, Sykova E. Mechanisms of neurogenic pulmonary edema development. *Physiol Res* 2008;**57**:499–506.
15. Smith WS, Matthay MA. Evidence for a hydrostatic mechanism in human neurogenic pulmonary edema. *Chest* 1997;**111**:1326–33.
16. Blessing WW, West MJ, Chalmers J. Hypertension, bradycardia, and pulmonary edema in the conscious rabbit after brainstem lesions coinciding with the A1 group of catecholamine neurons. *Circ Res* 1981;**49**:949–58.
17. Chen HI, Chai CY. Integration of the cardiovagal mechanism in the medulla oblongata of the cat. *Am J Physiol* 1976;**231**:454–61.
18. Darragh TM, Simon RP. Nucleus tractus solitarius lesions elevate pulmonary arterial pressure and lymph flow. *Ann Neurol* 1985;**17**:565–9.
19. Lin TY, Chang LY, Hsia SH, Huang YC, Chiu CH, Hsueh C, et al. The 1998 enterovirus 71 outbreak in Taiwan: pathogenesis and management. *Clin Infect Dis* 2002;**34**:S52–7.
20. McMinn PC. An overview of the evolution of enterovirus 71 and its clinical and public health significance. *FEMS Microbiol Rev* 2002;**26**:91–107.
21. Wu JM, Wang JN, Tsai YC, Liu CC, Huang CC, Chen YJ, et al. Cardiopulmonary manifestations of fulminant enterovirus 71 infection. *Pediatrics* 2002;**109**:e26.
22. Nakamura K, Sasaki S, Moriguchi J, Morinoto S, Miki S, Kawa T, et al. Central effects of endothelin and its antagonist on sympathetic and cardiovascular regulation in SHR-SP. *J Cardiovasc Pharmacol* 1999;**33**:876–82.
23. Mosqueda-Garcia R, Yates K, O'Leary J, Inagami T. Cardiovascular and respiratory effects of endothelin in the ventrolateral medulla of the normotensive rat. *Hypertension* 1995;**26**:263–71.
24. Koseki C, Imai M, Hirata Y, Yanagisawa M, Masaki T. Binding sites for endothelin-1 in rat tissues: an autoradiographic study. *J Cardiovasc Pharmacol* 1989;**13**:S153–4.
25. Niwa M, Kawaguchi T, Himeno A, Fujimoto M, Kurihara M, Yamashita K, et al. Specific binding sites for 125I-endothelin-1 in the porcine and human spinal cord. *Eur J Pharmacol* 1992;**225**:281–9.
26. Schmitz T, Chew LJ. Cytokines and myelination in the central nervous system. *ScientificWorldJournal* 2008;**8**:1119–47.
27. Mizuno T, Sawada M, Suzumura A, Marunouchi T. Expression of cytokines during glial differentiation. *Brain Res* 1994;**656**:141–6.
28. Cannella B, Raine CS. Multiple sclerosis: cytokine receptors on oligodendrocytes predict innate regulation. *Ann Neurol* 2004;**55**:46–57.
29. Speciale L, Roda K, Saresella M, Taramelli D, Ferrante P. Different endothelins stimulate cytokine production by peritoneal macrophages and microglial cell line. *Immunology* 1998;**93**:109–14.
30. Griffiths MJ, Ooi MH, Wong SC, Mohan A, Podin Y, Perera D, et al. In enterovirus 71 encephalitis with cardio-respiratory compromise, elevated interleukin1β, interleukin 1 receptor antagonist, and granulocyte colony-stimulating factor levels are markers of poor prognosis. *J Infect Dis* 2012;**206**:881–92.
31. Lin TY, Hsia SH, Huang YC, Wu CT, Change LY. Proinflammatory cytokine reactions in enterovirus 71 infections of the central nervous system. *Clin Infect Dis* 2003;**36**:269–74.