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Anomalous expression of chloride transporters in the sclerosed hippocampus of mesial temporal lobe epilepsy patients☆

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Abstract

The Na^+-K^+-Cl^- cotransporter 1 and K^+-Cl^- cotransporter 2 regulate the levels of intracellular chloride in hippocampal cells. Impaired chloride transport by these proteins is thought to be involved in the pathophysiological mechanisms of mesial temporal lobe epilepsy. Imbalance in the relative expression of these two proteins can lead to a collapse of Cl^- homeostasis, resulting in a loss of gamma-aminobutyric acid-ergic inhibition and even epileptiform discharges. In this study, we investigated the expression of Na^+-K^+-Cl^- cotransporter 1 and K^+-Cl^- cotransporter 2 in the sclerosed hippocampus of patients with mesial temporal lobe epilepsy, using western blot analysis and immunohistochemistry. Compared with the histologically normal hippocampus, the sclerosed hippocampus showed increased Na^+-K^+-Cl^- cotransporter 1 expression and decreased K^+-Cl^- cotransporter 2 expression, especially in CA2 and the dentate gyrus. The change was more prominent for the Na^+-K^+-Cl^- cotransporter 1 than for the K^+-Cl^- cotransporter 2. These experimental findings indicate that the balance between intracellular and extracellular chloride may be disturbed in hippocampal sclerosis, contributing to the hyperexcitability underlying epileptic seizures. Changes in Na^+-K^+-Cl^- cotransporter 1 expression seems to be the main contributor. Our study may shed new light on possible therapies for patients with mesial temporal lobe epilepsy with hippocampal sclerosis.

Key Words

neural regeneration; brain injury; mesial temporal lobe epilepsy; hippocampal sclerosis; sodium-potassium chloride cotransporter 1; potassium chloride cotransporter 2; gamma-aminobutyric acid; chloride ion; dentate gyrus; CA2 region; human; grants-supported paper; photographs-containing paper; neuroregeneration

Research Highlights

(1) Sclerosed hippocampi from mesial temporal lobe epilepsy patients were compared with histologically normal hippocampi, with the aim of analyzing changes in the expression of Na^+-K^+-Cl^- cotransporter 1 and K^+-Cl^- cotransporter 2.
(2) The balance of intracellular and extracellular chloride may be disturbed in hippocampal sclerosis through dysregulation of Na^+-K^+-Cl^- cotransporter 1 and K^+-Cl^- cotransporter 2 expression, which could contribute to hyperexcitability and seizures. The Na^+-K^+-Cl^- cotransporter 1 may be the main contributor.
(3) The findings may shed new light on possible therapies for mesial temporal lobe epilepsy with hippocampal sclerosis.

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INTRODUCTION

Epilepsy is a common disorder of the nervous system. It has been reported that about 22.5% of patients with epilepsy eventually develop drug-resistance[1]. Most of these refractory patients have mesial temporal lobe epilepsy with hippocampal sclerosis[2]. Therefore, further studies addressing the mechanisms of mesial temporal lobe epilepsy with hippocampal sclerosis may help us understand the underlying pathogenesis of drug-resistant epilepsy.

In the adult brain, gamma-aminobutyric acid is the primary inhibitory neurotransmitter. It triggers hyperpolarization of neurons through activation of chloride-permeable gamma-aminobutyric acid A receptors[3], which mediate the passive movement of chloride ions into the cell. The effect of gamma-aminobutyric acid on membrane potential can be altered by changes in the electrochemical gradient of chloride across the cell membrane[4-5]. Such changes have been reported in association with abnormal expression of cation-chloride cotransporters[6-8].

The Na^+-K^+-Cl^- cotransporter 1 and K^+-Cl^- cotransporter 2 are two important cation-chloride cotransporter proteins[9]. Various studies have shown a correlation between epileptogenesis and altered expression of Na^+-K^+-Cl^- cotransporter 1 and K^+-Cl^- cotransporter 2. For instance, spinal cord injury-induced attenuation of gamma-aminobutyric acid-ergic inhibition in spinal dorsal horn circuits was associated with downregulation of K^+-Cl^- cotransporter 2 in a rat[10]. Other studies have shown altered expression of Na^+-K^+-Cl^- cotransporter 1 and K^+-Cl^- cotransporter 2 in various animal models of epilepsy and in surgical specimens obtained from epilepsy patients[11-12]. Palma *et al* [13] found increased Na^+-K^+-Cl^- cotransporter 1 and decreased K^+-Cl^- cotransporter 2 expression in the subiculum of drug-resistant epilepsy patients. Other studies also attributed refractory seizures to increased Na^+-K^+-Cl^- cotransporter 1 expression[14-15].

The contribution of Na^+-K^+-Cl^- cotransporter 1 and K^+-Cl^- cotransporter 2 to seizures in patients with mesial temporal lobe epilepsy with hippocampal sclerosis has been investigated to some extent[13, 15]. However, the conclusions from the previous studies are not reliable because of the use of ipsilateral temporal neocortices as control tissue, despite the lack of evidence of equivalent Na^+-K^+-Cl^- cotransporter 1 and K^+-Cl^- cotransporter 2 expression in normal hippocampus and normal neocortex. Therefore, the possible changes in Na^+-K^+-Cl^- cotransporter 1 and K^+-Cl^- cotransporter 2 expression in the sclerosed hippocampus need further investigation.

In the current study, we used histologically normal hippocampi as a control tissue to enable us to obtain more reliable data on the changes of Na^+-K^+-Cl^- cotransporter 1 and K^+-Cl^- cotransporter 2 expression in the sclerosed hippocampus from patients with mesial temporal lobe epilepsy.

RESULTS

Quantitative analysis of participants

A total of 45 epilepsy patients were consecutively enrolled in this study. Among them, 13 patients with hippocampal sclerosis met the inclusion criteria for the hippocampal sclerosis group, and six patients without hippocampal sclerosis met the inclusion criteria for the control group. These 19 patients were included in the final analysis.

Baseline data of participants

According to pre-operative MRI, intraoperative depth electrode recording, and post-operative pathological examination, the patients were divided into two groups: hippocampal sclerosis group and histologically normal group (control group). The characteristics of the patients are summarized in Table 1. In the hippocampal sclerosis group, the patients had a definite aura before seizures, such as epigastric symptoms and sensation of fear. Classic hippocampal sclerosis was demonstrated by MRI and post-operative pathological examination. Spontaneous epileptiform discharges in the hippocampus were confirmed by depth electrode recording. Patients in the control group had different foci near the hippocampus (Table 1). Hippocampi in this group were initially thought to have foci, but following surgical removal they were found to be histologically normal. Because of the strict criteria for inclusion in the control group, few epilepsy patients met the criteria. Therefore, the age and gender were not well matched between the two groups. However, the differences between them were not obvious.

Na^+-K^+-Cl^- cotransporter 1 and K^+-Cl^- cotransporter 2 expression in the hippocampus

Western blot analysis was used to compare the expression of Na^+-K^+-Cl^- cotransporter 1 and K^+-Cl^- cotransporter 2 in sclerosed and control hippocampi. Equal amounts of tissue were used from the two groups.

Table 1 Clinical findings of enrolled patients

Case No.	Gender	Age at resection	Age at onset	FC	Aura	CP	SG	Pathology of resected hippocampus	Other definite cause
1	F	30y	23y	+	Epigastric aura	+	+	HS Grade IV	-
2	M	16y	6y	+	Epigastric aura	+	+	HS Grade IV	-
3	M	29y	7y	-	Epigastric aura	+	+	HS Grade IV	-
4	F	19y	9y	-	Epigastric aura	+	+	HS Grade IV	-
5	M	15y	10y	+	Epigastric aura	+	+	HS Grade IV	-
6	M	26y	10y	+	Epigastric aura	+	+	HS Grade IV	-
7	M	19y	13y	+	Epigastric aura	+	+	HS Grade IV	-
8	M	13y	9y	-	Fear aura	+	+	HS Grade IV	-
9	M	7y5m	6y9m	+	Epigastric aura	+	+	HS Grade IV	-
10	M	18y	14y	-	Fear aura	+	-	HS Grade IV	-
11	M	21y	17y	+	Epigastric aura	+	+	HS Grade IV	-
12	F	36y	17y	+	Epigastric aura	+	-	HS Grade IV	-
13	F	28y	23y	-	Fear aura, epigastric aura	+	+	HS Grade IV	-
1C	F	57y	56y	-	-	+	-	Histologically normal	CCM
2C	M	51y	-	-	-	-	-	Histologically normal	Temporal glioma
3C	F	15y	3y	-	-	+	+	Histologically normal	Temporal glioma
4C	F	24y	23y	-	-	+	+	Histologically normal	DNT
5C	F	12y11m	1y	-	-	+	+	Histologically normal	Pachygyria
6C	M	18y	11y	-	-	+	+	Histologically normal	DNT

M: Male; F: female; y: year; m: month; FC: febrile convulsion; CP: complex partial seizure; SG: secondarily generalized seizure; HS: hippocampal sclerosis; CCM: cerebral cavernous hemangioma; DNT: dysembryoplastic neuroepithelial tumor; HS Grade IV: gliosis and neuronal loss in CA1 and CA4 with sparing of CA2-according to modified Wyler classification[16]; C: control group.

Na^+-K^+-Cl^- cotransporter 1 appeared as a single band of approximately 170 kDa, whereas K^+-Cl^- cotransporter 2 was seen as a band of 140 kDa (Figure 1).

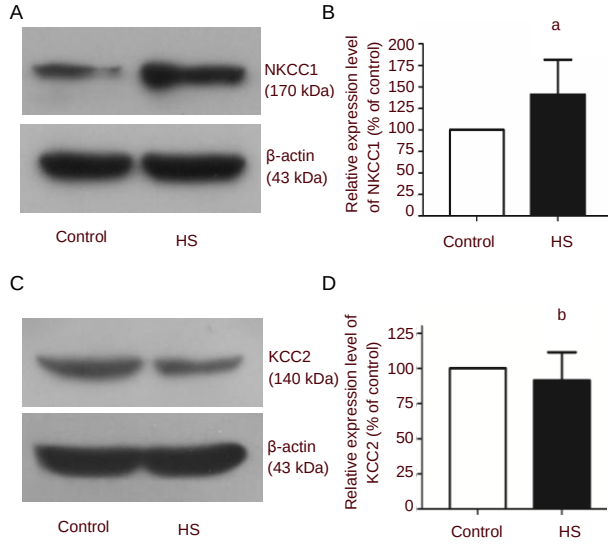

Figure 1 Western blot analysis of NKCC1 (A, B) and KCC2 (C, D) expression in the hippocampus.

β-actin is shown as a loading control. Intergroup differences were compared using independent sample *t*-tests. $^aP < 0.01$, $^bP < 0.05$, *vs*. control. Data are expressed as mean ± SD ($n = 39$). HS: Hippocampal sclerosis; NKCC1: Na^+-K^+-Cl^- cotransporter 1; KCC2: K^+-Cl^- cotransporter 2.

Densitometric analysis of the signal intensity was normalized to β-actin and revealed a 1.4-fold increase in Na^+-K^+-Cl^- cotransporter 1 expression ($P < 0.01$) and a 10% decrease of K^+-Cl^- cotransporter 2 expression ($P < 0.05$) in most samples from sclerosed hippocampus compared with histologically normal tissue. The change in Na^+-K^+-Cl^- cotransporter 1 expression was more pronounced than that of K^+-Cl^- cotransporter 2.

We also used immunohistochemistry to investigate alterations of Na^+-K^+-Cl^- cotransporter 1 and K^+-Cl^- cotransporter 2 expression in the different subfields of the hippocampus. Compared with the histologically normal hippocampus, most neurons in the majority of surgically resected sclerosed hippocampi demonstrated stronger positive immunoreactivity for Na^+-K^+-Cl^- cotransporter 1 in all subfields, especially in area CA2 and the dentate gyrus ($P < 0.01$, Figure 2). Na^+-K^+-Cl^- cotransporter 1 expression was increased by 50% in CA2 and dentate gyrus of sclerosed hippocampi. Additionally, the sclerosed hippocampi showed slightly decreased K^+-Cl^- cotransporter 2 expression in all subfields ($P < 0.05$, Figure 3).

DISCUSSION

Mesial temporal lobe epilepsy is now recognized as the most common epilepsy syndrome in adults, and hippocampal sclerosis is seen in 50–70% of cases[17].

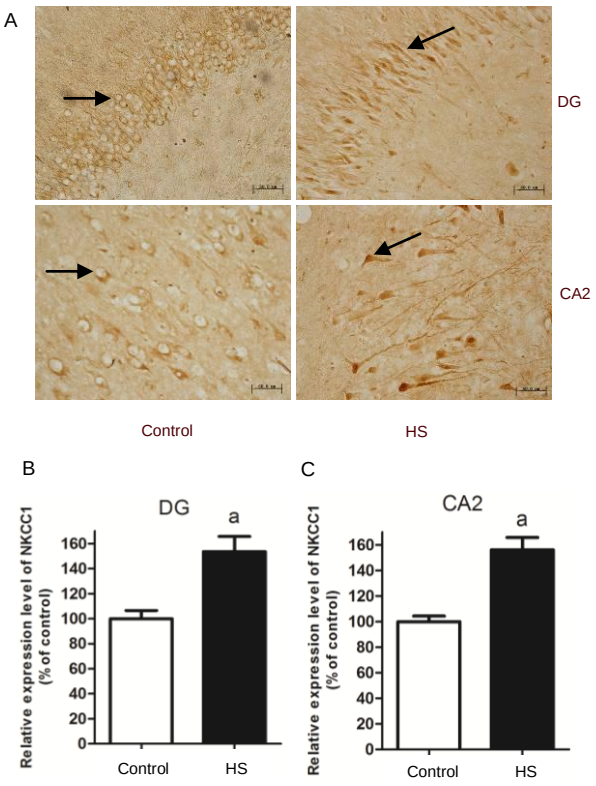

Figure 2 Na^+-K^+-Cl^- cotransporter 1 (NKCC1) expression in the CA2 and dentate gyrus (DG) of the sclerosed hippocampus (HS) of patients with mesial temporal lobe epilepsy.

(A) Immunohistochemistry analysis of NKCC1 expression in CA2 and the DG of the hippocampus. Images were photographed with a BX51 biological microscope. The cells marked with arrows in A are typical NKCC1-positive cells. Scale bars: 50 μm.

(B, C) The absorbance analysis of NKCC1 immunoreactivity in the DG and CA2 regions in the HS group (n = 13) and histological normal group (control, n = 6). $^aP < 0.01$, *vs*. control, independent sample *t*-test. Data are expressed as mean ± SD.

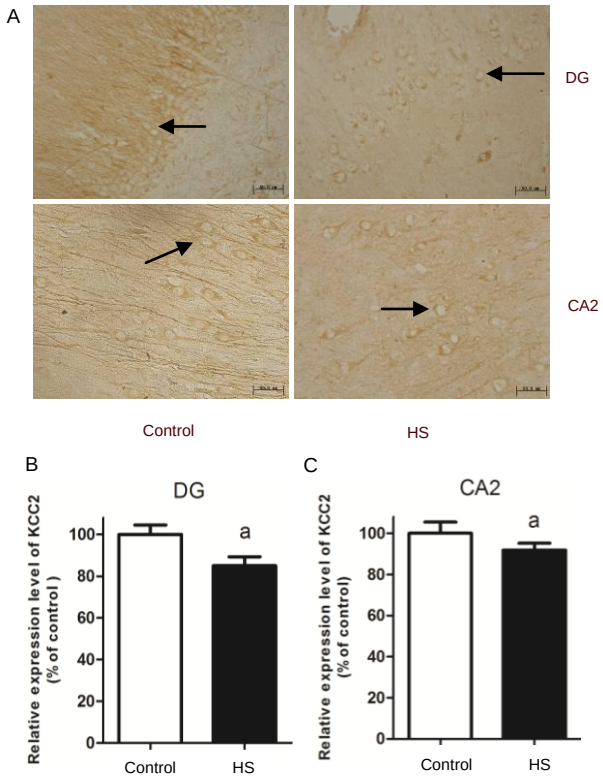

Figure 3 K^+-Cl^- cotransporter 2 (KCC2) expression in the CA2 and dentate gyrus (DG) of the sclerosed hippocampus (HS) of patients with mesial temporal lobe epilepsy.

(A) Immunohistochemistry analysis of KCC2 expression in the CA2 and DG of the hippocampus. Images were photographed with a BX51 biological microscope. The cells marked with arrows in A are typical KCC2-positive cells. Scale bars: 50 μm.

(B, C) The absorbance analysis of KCC2 immunoreactivity in DG and CA2 in the HS group (n = 13) and histological normal group (control, n = 6). $^aP < 0.05$, *vs*. control, independent sample *t*-test. Data are expressed as mean ± SD.

The role of hippocampal sclerosis in epileptogenesis is not fully understood. We focused on the regulation of intracellular chloride by Na^+-K^+-Cl^- cotransporter 1 and K^+-Cl^- cotransporter 2 in the sclerosed hippocampus of patients with mesial temporal lobe epilepsy, because increased intracellular chloride concentrations is known to cause excitatory actions of gamma-aminobutyric acid[18].

In the present study, the expression of Na^+-K^+-Cl^- cotransporter 1 and K^+-Cl^- cotransporter 2 in hippocampal specimens was detected by immunohistochemistry and western blot analysis. We found upregulation of Na^+-K^+-Cl^- cotransporter 1 and downregulation of K^+-Cl^- cotransporter 2 in sclerosed hippocampi, especially in the CA2 and dentate gyrus subfields.

In the central nervous system, Na^+-K^+-Cl^- cotransporter 1 and K^+-Cl^- cotransporter 2 are important proteins that regulate intracellular chloride concentrations[19]. Under physiological conditions, Na^+-K^+-Cl^- cotransporter 1 promotes the accumulation of chloride inside the cell, while K^+-Cl^- cotransporter 2 mainly extrudes chloride ions from the cell. The net effect of their actions is to sustain relatively low intracellular chloride concentrations. Imbalance in their function will lead to a collapse of the normal chloride gradient, preventing gamma-aminobutyric acid from generating inhibitory hyperpolarizing potentials and even resulting in gamma-aminobutyric acid-ergic excitation. Some researchers have found that gamma-aminobutyric acid-ergic excitation may be the cause of epileptiform discharges evoked in human surgical specimens[20-21].

Therefore, expressional changes of Na^+-K^+-Cl^- cotransporter 1 and K^+-Cl^- cotransporter 2 may play an important role in the mechanism of epileptogenesis.

In 2006, Palma *et al* [13] reported that mRNA expression of Na^+-K^+-Cl^- cotransporter 1 was upregulated in sclerosed hippocampi of patients with drug-resistant epilepsy. Recently, Sen *et al* [15] also found increased levels of Na^+-K^+-Cl^- cotransporter 1 in CA2 and dentate gyrus of the sclerosed hippocampus. Both studies regarded this as the cause of repeated seizures. Our findings are consistent with these studies. However, previous studies used neocortices or post-mortem specimens as control tissue. The use of temporal neocortex is problematic because it is not derived from the same tissues as hippocampus, *i.e*. the expression and function of Na^+-K^+-Cl^- cotransporter 1 may not be the same. The use of post-mortem tissue carries the problem of protein loss, even though the post-mortem interval was strictly defined. Thus, these previously used controls may introduce some errors that confound the results. In this study, we avoid these problems and produce more reliable findings by using histologically normal hippocampi as controls.

The dentate gyrus has long been considered as the region responsible for epileptogenesis in temporal lobe epilepsy[22], but the mechanism is not fully understood. Our finding of increased Na^+-K^+-Cl^- cotransporter 1 expression in the dentate gyrus may contribute to our understanding of the role of the dentate gyrus in epileptogenesis. Whereas the normal dentate gyrus has a high threshold for seizure generation, the sclerosed hippocampus may be prone to spontaneous epileptiform discharges because of high intracellular chloride concentrations caused by elevated Na^+-K^+-Cl^- cotransporter 1 expression.

Upregulation of Na^+-K^+-Cl^- cotransporter 1 expression was also detected in area CA2. The role of the CA2 region in epileptogenesis has not been fully explored. It is generally accepted that the CA2 region is not involved in the classic trisynaptic circuit of the hippocampus, and it does not seem to contribute to hippocampal neuronal transmission[23]. Why was Na^+-K^+-Cl^- cotransporter 1 upregulated in this non-essential region? We presume that most of the neurons in CA1 and CA3 regions die because of hippocampal sclerosis, so the trisynaptic circuit is destroyed and the neural network is reorganized. After reorganization, pyramidal neurons in CA2 may take over most of the responsibility for the transfer of signals through the hippocampus. Some recent studies have also shown that CA2 may participate in information transmission in the hippocampal circuit[24]. Thus, the observed upregulation of Na^+-K^+-Cl^- cotransporter 1 expression in CA2 of the sclerosed hippocampus might also contribute to epileptogenesis.

Some studies have reported that K^+-Cl^- cotransporter 2 is critically involved in epileptogenesis in the cortex[14, 25-26], but the role of K^+-Cl^- cotransporter 2 in the hippocampus has not previously been mentioned. While we demonstrated a downregulation of K^+-Cl^- cotransporter 2 in the hippocampus in this study, the change was smaller than that of Na^+-K^+-Cl^- cotransporter 1. This suggests that Na^+-K^+-Cl^- cotransporter 1 plays a more critical role in regulating intracellular chloride in the hippocampus.

In the present study, the sample size of the control group was small because of the strict enrolment criteria. It is very difficult to obtain a fresh but normal hippocampus, and only a few epilepsy patients completely met the inclusion criteria. However, we think this sample size was sufficient to illustrate the problem of this study.

According to our results, increased Na^+-K^+-Cl^- cotransporter 1 and decreased K^+-Cl^- cotransporter 2 expression existed in the CA2 and dentate gyrus subfields of the sclerosed hippocampus. This might contribute to hyperexcitability that leads to seizures. Changes in Na^+-K^+-Cl^- cotransporter 1 expression may be the main contributor. The commonly used and generally well-tolerated diuretic bumetanide, an Na^+-K^+-Cl^- cotransporter 1 antagonist[27], may provide a promising therapy for mesial temporal lobe epilepsy, possibly even for drug-resistant forms.

MATERIALS AND METHODS

Design

A case-control study.

Time and setting

Experiments were performed at the Laboratory of Neurology, the First Affiliated Hospital, Sun Yat-sen University, China from April 2010 to July 2011.

Subjects

All of the cases were inpatients of the First Affiliated Hospital of Sun Yat-sen University and Guangdong 999 Brain Hospital. All procedures were conducted according to the *Administrative Regulations on Medical Institutions*,

formulated by the State Council of China[28], and institutional approval was obtained from the hospital ethics committees. The patients were informed of the experimental protocol and risks, and informed consent was obtained from each patient.

Hippocampal sclerosis group

The subjects in this group had mesial temporal lobe epilepsy with hippocampal sclerosis. The inclusion criteria are as follows: (1) clinical diagnosis of mesial temporal lobe epilepsy according to the criteria proposed by the International League Against Epilepsy[29]; (2) the patients underwent resective surgery because of drug-resistance; and (3) the post-operative pathological examination confirmed sclerosis of the resected hippocampus.

Control group

The inclusion criteria for the controls are as follows: (1) The patient was diagnosed with symptomatic epilepsy with a focus near the hippocampus demonstrated by MRI and the focus was identified as the epileptogenic zone by depth electrode recording; (2) no aura before seizures was described in the clinical presentation; (3) the hippocampus could not be confirmed as normal by pre-operative MRI and was surgically removed with approval of the patient, but was identified as normal on pathological examination by two independent neuropathologists.

Methods

Specimen preparation

After excision, western blot analysis was performed on fresh hippocampal tissue that was frozen in liquid nitrogen. Immunohistochemistry was performed on fresh hippocampal tissue that was fixed with 4% paraformaldehyde.

Western blot analysis

Membrane proteins were extracted from each hippocampus according to the protocol supplied with the Membrane Protein Extraction Kit (Thermo Scientific Pierce, Rockford, IL, USA). Protein concentrations were determined using the Micro BCA™ Protein Assay Reagent (Thermo Scientific Pierce). For individual western blot experiments, 30 or 60 μg of protein per lane was electrophoretically separated and transferred to an immobilon polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked in 5% fresh non-fat milk for 60 minutes at room temperature and incubated with primary antibodies (Na^+-K^+-Cl^- cotransporter 1, goat-anti-human, polyclonal IgG, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA; K^+-Cl^- cotransporter 2, goat-anti-human, polyclonal IgG, 1:100; Santa Cruz Biotechnology) overnight at 4°C, followed by washes in Tris-buffered saline with Tween. The membrane was incubated with horseradish peroxidase-conjugated secondary antibody (donkey-anti-goat IgG, 1:1 000; Santa Cruz Biotechnology) for 2 hours at room temperature. Immunodetection of proteins by chemiluminescence was followed by exposure to X-ray film. Rabbit-anti-human β-actin monoclonal antibody (1:2 000; Cell Signaling Technology, Boston, MA, USA) and horseradish peroxidase-conjugated secondary antibody (polyclonal IgG, donkey-anti-rabbit, 1:400; Thermo Scientific Pierce) were used to detect β-actin expression.

Immunohistochemistry

Frozen coronal sections (10 μm thickness) were cut on a cryostat and thaw-mounted onto polylysine-coated slides. They were thoroughly washed with 0.01 M PBS and then incubated in 1% H_2O_2 to block endogenous peroxidase activity. After being washed with PBS, the sections were incubated with a blocking solution (2% bovine serum albumin, 0.3% Triton X-100, and 5% normal pig serum in Tris-buffered saline) for 1 hour at room temperature and directly transferred into the primary antibody (Na^+-K^+-Cl^- cotransporter 1, goat-anti-human, polyclonal IgG, 1:40, Santa Cruz Biotechnology; K^+-Cl^- cotransporter 2, goat-anti-human, polyclonal IgG, 1:50, Santa Cruz Biotechnology) overnight at 4°C. After being rinsed with PBS, the sections were placed in peroxidase-labeled secondary antibody (donkey anti-goat IgG, 1:50; Bioworld, Minneapolis, MN, USA) for 1 hour at room temperature. They were then washed with PBS again and incubated in 3,3'-diaminobenzidine (DAKO, Glostrup, Denmark) for 3 minutes[30]. Finally, sections were washed, treated with dehydrated alcohol and dimethyl benzene, and cover-slipped for analysis.

Image analysis and quantification

Images were captured under a biological microscope (BX51, Olympus, Tokyo, Japan) and analyzed using image analysis software (NIH Image J 1.42, Bethesda, MD, USA). For western blot analysis, densitometry analysis for the quantification of bands was performed as described on the NIH Image J analysis website (http://rsb.info.nih.gov/ij/)[31]. Band intensities were measured in duplicate for each homogenate. Reactive optical densities of specific reactive bands corrected for background were averaged per sample and the means were used for statistical analysis.

For immunohistochemistry, the absorbance of immunostained cells was chosen as the concentration of Na^+-K^+-Cl^- cotransporter 1 or K^+-Cl^- cotransporter 2. The analysis was performed as described previously[32]. Six sections per sample and six cells per region were analyzed. Cells were chosen randomly for analysis from each region of each section. The mean absorbance value was obtained by averaging absorbance values of analyzed cells in each region. The background absorbance of each section was measured and subtracted from the mean absorbance. A single mean absorbance value was then obtained for each region of each section. As the analysis was carried out on six sections per sample, six mean absorbance values were obtained for each region in each sample. Last, the six mean absorbance values were averaged per sample and the means were used for statistical analysis.

Statistical analysis

All numerical values were expressed as mean ± SD. All statistical analyses were performed using SPSS 16.0 software (SPSS, Chicago, IL, USA). The data in this study were normally distributed. Comparisons of two groups were carried out using independent sample *t*-tests. $P < 0.05$ was considered statistically significant.

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Author contributions: Xiaodong Cai and Libai Yang were in charge of data integration, analysis and paper writing. Liemin Zhou obtained funding and was in charge of study design and paper review. Dan Zhu and Qiang Guo were responsible for all aspects of patient recruitment and clinical diagnostic assessment. Jueqian Zhou, Ziyi Chen and Shuda Chen were in charge of data analysis and statistical processing. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Ethical approval: The experiment was approved by the Ethics Committees of the First Affiliated Hospital of Sun Yat-sen University and Guangdong 999 Brain Hospital, Guangzhou, China.

Author statements: The manuscript is original, has not been submitted to and is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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