Int J Clin Exp Med 2016;9(7):13979-13985
www.ijcem.com /ISSN:1940-5901/IJCEM0026588

Original Article

Expressions of EF-Tu$_{mt}$ and EF-Ts$_{mt}$ in epileptogenic zone of patients with refractory temporal lobe epilepsy

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Received February 24, 2016; Accepted June 4, 2016; Epub July 15, 2016; Published July 30, 2016

Abstract: Objective: To examine the expressions of mitochondrial translation elongation factors Tu (EF-Tu$_{mt}$) and Ts (EF-Ts$_{mt}$) in the epileptogenic zone of patients with refractory temporal lobe epilepsy. Methods: Epileptogenic zone resection specimens were obtained from 20 patients with refractory temporal lobe epilepsy. Control samples were temporal lobe cortex tissues derived from 6 non-epileptic patients with brain trauma. Histological examination was performed using hematoxylin and eosin (H&E) staining. EF-Tu$_{mt}$ and EF-Ts$_{mt}$ expression patterns were evaluated using immunohistological analysis. Ultrastructural alterations were determined by transmission electron microscopy. Subcellular localization of target proteins was examined by immune electron microscopy. Results: Pathological changes and ultrastructural alterations were observed in all epileptogenic zone specimens taken from the patients with refractory temporal lobe epilepsy. Immunostaining indicated that the number of EF-Tu$_{mt}$-positive and EF-Ts$_{mt}$-positive cells in epilepsy group was significantly higher than those in control subjects (50.99 ± 9.41 vs. 27.87 ± 3.46, P<0.05 and 53.00 ± 9.40 vs. 27.94 ± 7.05, P<0.05 respectively). Electron microscopy analysis further revealed that the number of EF-Tu$_{mt}$-positive and EF-Ts$_{mt}$-positive particles in epilepsy patients was also higher than those in the controls (112.76 ± 20.79 vs. 32.67 ± 10.73, P<0.01; 111.48 ± 19.55 vs. 33.50 ± 8.62, P<0.01). In addition, both EF-Tu$_{mt}$ and EF-Ts$_{mt}$ were predominantly localized within mitochondria. Conclusions: EF-Tu$_{mt}$ and EF-Ts$_{mt}$ are up-regulated in the epileptogenic zone of patients with refractory temporal lobe epilepsy, implying that these proteins may contribute to the pathogenesis and progression of epilepsy.

Keywords: EF-Tu$_{mt}$, EF-Ts$_{mt}$, epileptogenic zone, refractory temporal lobe epilepsy

Introduction

Temporal lobe epilepsy (TLE) is a chronic neurological disorder characterized by recurrent seizures that originate in the temporal lobe of the human brain. TLE is one of the most common types of epilepsy and may develop into the refractory form of TLE [1-3]. Multiple antiepileptic drugs (AEDs) have been developed and are available for safe and effective use in patients with refractory TLE, but many of these patients develop resistance to AED therapy. Moreover, the pathogenesis of this disease and AED resistance remains unclear.

Mitochondria, found in most eukaryotic cells, generate most of the cell's supply of adenosine triphosphate (ATP), which is an important source of the body's chemical energy. The mitochondrial translation elongation factors Tu (EF-Tu$_{mt}$) and Ts (EF-Ts$_{mt}$) are critically involved in mitochondrial translation, and their expression levels can reflect mitochondrial function. The GTPase molecule EF-Tu$_{mt}$ is responsible for delivering the aminoacylated tRNA (aa-tRNA) to the A site (acceptor site) of the ribosome during the elongation step of translation, subsequently forming the EF-Tu·GTP·aa-tRNA complex [4]. When the complex dissociates, the EF-Tu$_{mt}$ is released with the exchange of GDP for GTP. EF-Ts$_{mt}$ promotes the exchange of GDP for GTP through the formation of a complex with EF-Tu·Ts [5]. Most of the EF-Tu$_{mt}$ proteins are found on the mitochondrial inner membrane, where most mitochondrial ribosomes are located, and function as a chaperone in the mitochondrial protein quality control processes [6]. Although mitochondrial dysfunction has been linked to

epilepsy, the potential involvement of mitochondrial Tu (EF-Tu$_{mt}$) and Ts (EF-Ts$_{mt}$) in this disease has not yet been clarified [7, 8].

In the current study, we compared the histological and ultrastructural alterations of brain tissues between patients with refractory TLE and non-TLE control subjects. Furthermore, we determined the expression patterns of EF-Tu$_{mt}$ and EF-Ts$_{mt}$ in the epileptogenic zone of the brain sections. Our findings may provide valuable insights into understanding the pathogenesis and progression of refractory temporal lobe epilepsy.

Materials and methods

Reagents

Mouse anti-human anti-EF-Ts$_{mt}$ IgG and anti-EF-Tu$_{mt}$ IgG primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The UltraSensitive ™ SP kit for immunostaining was obtained from Maxim Biotech (Fuzhou, China). Goat anti-mouse gold-conjugated secondary antibody was purchased from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China). The 3,3' diaminobenzidine (DAB) kit was purchased from Changchun Dingguo Bio Co. (Changchun, China).

Patients

A total of 20 patients with refractory TLE who underwent resection of the epileptogenic zone at the First Hospital of Jilin University (Changchun, Jilin, China) were enrolled in the study. All of these patients met the classification criteria of epileptic seizures and syndromes as determined by the International League Against Epilepsy (1981 and 1989). Among these patients, 11 were male and 9 were female, with ages ranging from 16 to 58 years and an average age of 27.70 ± 11.14 years. The disease course among the total 20 patients varied from 2 to 29 years, with an average duration of 9.45 ± 7.67 years. All patients received routine electroencephalography (EEG), long-term video-EEG, electrocorticogram (ECoG), and brain magnetic resonance imaging (MRI). All patients had undergone treatment with two or more first-line AEDs (including phenytoin sodium, carbamazepine, valproate, and phenobarbital) for more than 2 years, but the symptoms were uncontrolled with seizures occurring over four times per month.

Control brain tissues were obtained from 6 patients who underwent debridement for brain trauma injury. The controls included 4 males and 2 females, with ages ranging from 16 to 55 years and an average age of 36.83 ± 16.31 years. None of the controls had a current diagnosis or familial heredity history of epilepsy. Histological examinations of the brain tissues confirmed normal structure.

All study participants provided informed consent. Ethical approval for this study was granted by the First Hospital of Jilin University (Changchun, Jilin, China).

Collection of brain tissue samples

Tissue samples were obtained from patients with refractory temporal lobe epilepsy who underwent resection of the epileptogenic zone. Control samples of temporal lobe cortex tissues were obtained from non-epileptic patients with traumatic brain injury.

Histological examination

Brain tissue samples were rinsed with normal saline, then minced into small pieces, and fixed in 4% paraformaldehyde. For histological studies, the samples were stained with hematoxylin and eosin (H&E) and examined under light microscope.

Immunohistochemical analysis

For immunohistochemical analysis, the tissue samples were embedded in paraffin, sectioned into 5-μm thickness, dewaxed, hydrated, and washed. The sections were then heated in citrate buffer (0.01 M, pH 6.0) at 100°C for antigen retrieval and incubated with 10% goat serum to block nonspecific binding sites. Then, the sections were immunostained with primary antibodies for EF-Ts$_{mt}$ (1:100 dilution) and EF-Tu$_{mt}$ (1:100 dilution) respectively, at 4°C overnight. After three washes with phosphate-buffered saline (PBS), staining was detected by incubation with secondary antibodies (1:100 dilution) and DAB reagent. Subsequently, all sections were double-stained with hematoxylin, dehydrated in an ethanol series, cleared by incubating in xylene, mounted, and visualized under a light microscope at 400 × magnification. Five fields of vision were randomly selected from each section and used to make photomicrographs representative of the overall sam-

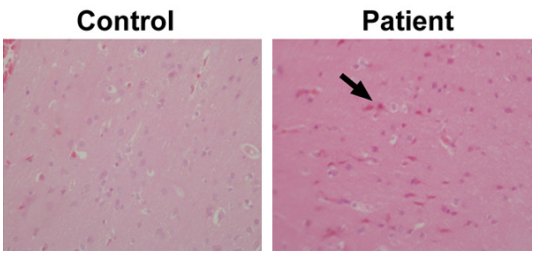

Figure 1. Histological examination of brain tissues derived from control subjects and patients with refractory temporal lobe epilepsy. Samples were stained with H&E; nuclei stained blue and cytoplasm stained pink. Control samples were temporal lobe cortex tissues derived from non-epileptic patients with brain trauma. Experimental samples were epileptogenic zone tissues derived from patients with refractory temporal lobe epilepsy. Arrow indicates the neuronal degeneration and small blood vessel formation. Magnification, × 400.

ple. The immuno-positive cells were counted in each selected field of vision, and used to calculate the average number for each section.

Transmission electron microscopy (TEM) and immune electron microscopy analysis

For TEM analysis, the brain tissue samples were fixed in 4% glutaraldehyde, and post-fixed in 2% osmic acid. After dehydration through an acetone gradient, the samples were immersed with a mixture of epoxy resin/acetone (1:1) and embedded with a mixture of Epon812 (Epoxiaquivalentgewicht 145-160), DDSA (Dodecenylsuccinic Anhydride), MNA (Methyl Nadic Anhydride), DMP30 (dimethylaminomethyt phenol) at 60°C. Ultrathin sections of 50-70 nm were placed onto 200 μm mesh grids and incubated with 1% hydrogen peroxide for 10-60 min. After washing with distilled water, the samples were incubated with normal goat serum for 30-60 min at room temperature to block non-specific binding sites, rinsed with PBS, and stained with either primary antibodies for EF-Ts$_{mt}$ (1:100 dilution) and EF-Tu$_{mt}$ (1:100 dilution), respectively (for experimental samples) or with PBS alone (for negative control samples), at room temperature for 1 h, followed by overnight incubation at 4°C. After PBS washing, the samples were incubated with gold-conjugated secondary antibody (1:100 dilution) at room temperature for 10-60 min. Sections were stained with 5% uranyl acetate and citrate uranium. Images were obtained under TEM at 2000 to 20000 × magnification. Five fields of vision were randomly selected from each section to assess the ultrastructural alterations that were present in the epileptogenic zone. The number of particles was counted per field of vision, and the average number of particles was calculated for each section.

Statistical analysis

Data were presented as means ± standard deviation (SD). For statistical analysis, the SPSS13.0 statistical software package was used (SPSS Inc., Chicago, IL, USA). Statistical significance between different groups was determined using the *t*-test. A *p*-value of <0.05 was set as the threshold to indicate statistical significance. Diagrams were plotted using SPSS13.0 and Microsoft Office Excel 2003 software.

Results

Histological alterations in the epileptogenic zone of patients with refractory temporal lobe epilepsy

The brain tissues obtained from control subjects showed normal cell distribution and structure (**Figure 1**). The epileptogenic zone of patients with refractory temporal lobe epilepsy showed pathological alterations, including disturbance of cortical layers, unevenly distributed neurons, appearance of immature neurons, nuclear vacuolation, reduced cytoplasm/nuclei ratio, neuronal degeneration, small blood vessel growth and congestion, glial cell proliferation, lymphocyte and plasma cell infiltration, and the formation of lymphocyte sleeves (**Figure 1**).

Immunohistological alterations in the epileptogenic zone of patients with refractory temporal lobe epilepsy

As shown in **Figure 2**, EF-Tu$_{mt}$ and EF-Ts$_{mt}$ expression in temporal lobe cortex tissues derived from the control brain trauma patients was lower than that observed in the patients with refractory temporal lobe epilepsy. Moreover, the number of EF-Tu$_{mt}$- or EF-Ts$_{mt}$-positively stained neurons and glial cells was significantly higher in the epileptogenic zone tissues (P<0.05, **Table 1**). These data suggest that EF-Tu$_{mt}$ and EF-Ts$_{mt}$ might be up-regulated in the epileptogenic zone of patients with refractory temporal lobe epilepsy.

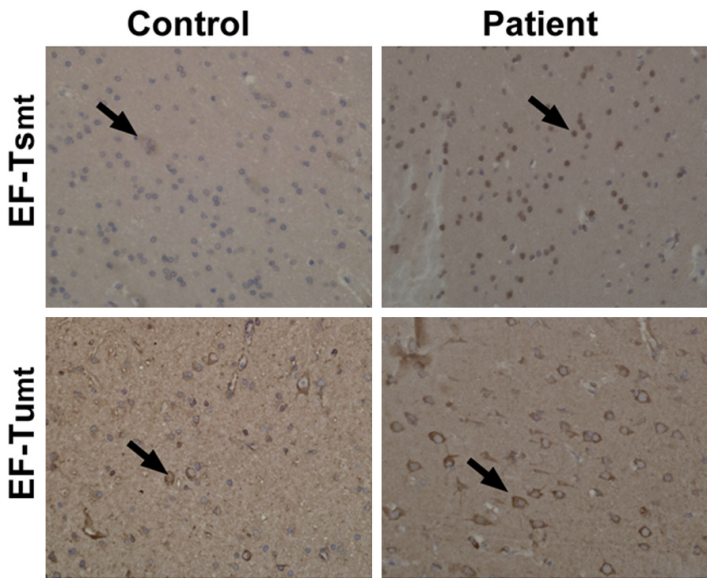

Figure 2. Immunohistological examination of the EF-Tu$_{mt}$ and EF-Ts$_{mt}$ expression in brain tissues derived from control subjects and patients with refractory temporal lobe epilepsy. Control samples were temporal lobe cortex tissues derived from non-epileptic patients with traumatic brain injury. Experimental samples were epileptogenic zone tissues derived from patients with refractory temporal lobe epilepsy. Samples were stained with anti-EF-Ts$_{mt}$ or anti-EF-Tu$_{mt}$ antibodies. Arrows indicate the positively stained cells. Magnification, × 400.

Table 1. Difference in average numbers of EF-Tu$_{mt}$- or EF-Ts$_{mt}$-positive cells in brain tissues from epileptics and controls, as determined by immunohistochemistry

Group	Factor	Number of positive cells	*t*	*p* ($\bar{X}$ ± SD)
Controls	EF-Tu$_{mt}$	27.87 ± 3.46	2.28	<0.05
Epileptics	EF-Tu$_{mt}$	50.99 ± 9.41		
Controls	EF-Ts$_{mt}$	27.94 ± 7.05	2.33	<0.05
Epileptics	EF-Ts$_{mt}$	53.00 ± 9.40		

Ultrastructural alterations in the epileptogenic zone of patients with refractory temporal lobe epilepsy

TEM analysis confirmed the neuronal degradation, necrosis, nuclear condensation, abnormal nuclear membrane structure, and accumulative vacuolation observed in the epileptogenic zone of patients with refractory temporal lobe epilepsy (**Figure 3**). Moreover, the features of mitochondrial swelling, vacuolation, mitochondrial cristae fragmentation, astrocyte swelling, cytoplasmic hypervacuolization, condensation, and margination of nuclear chromatin, nuclear envelope degradation were observed. Collectively, these findings demonstrated that the epileptogenic zone of patients with refractory temporal lobe epilepsy undergo ultrastructural changes that may be related to the pathogenic process of the disease.

Localization of EF-Tu$_{mt}$ and EF-Ts$_{mt}$

As shown by **Figure 4**, immune electron microscopy detection of the EF-Tu$_{mt}$- or EF-Ts$_{mt}$-positive gold particles showed that both EF-Tu$_{mt}$- and EF-Ts$_{mt}$-positive cells were present in the cytoplasm as well as in the mitochondria, especially in the latter. In addition, the epileptogenic zone tissues from patients with refractory temporal lobe epilepsy showed a significantly higher number of EF-Tu$_{mt}$- or EF-Ts$_{mt}$-positive gold particles than the tissue from the controls (P<0.01, **Figure 4** and **Table 2**). These observations further suggested the up-regulation of EF-Tu$_{mt}$ and EF-Ts$_{mt}$, especially within the mitochondria, in the epileptogenic zone of patients with refractory temporal lobe epilepsy.

Discussion

Mesial temporal sclerosis is accepted as the most common cause of refractory temporal lobe epilepsy and the condition usually presents with particular histological alterations, such as neuronal loss, hippocampal atrophy, and glial cell proliferation [9, 10]. In the current study, we observed histological and ultrastructural alterations in the epileptogenic zone of patients with refractory temporal lobe epilepsy that are consistent with the neuropathologic findings reported previously [9, 11].

Emerging evidence suggests that mitochondrial dysfunction and ultrastructural damage are closely associated with seizures in the rodent model of epilepsy [12, 13]. Moreover, increased expression of mitochondrial respiratory enzymes as well as of mitochondrial activity were observed in the brains of activated epilepsy-prone EI mice, which is a widely used rodent

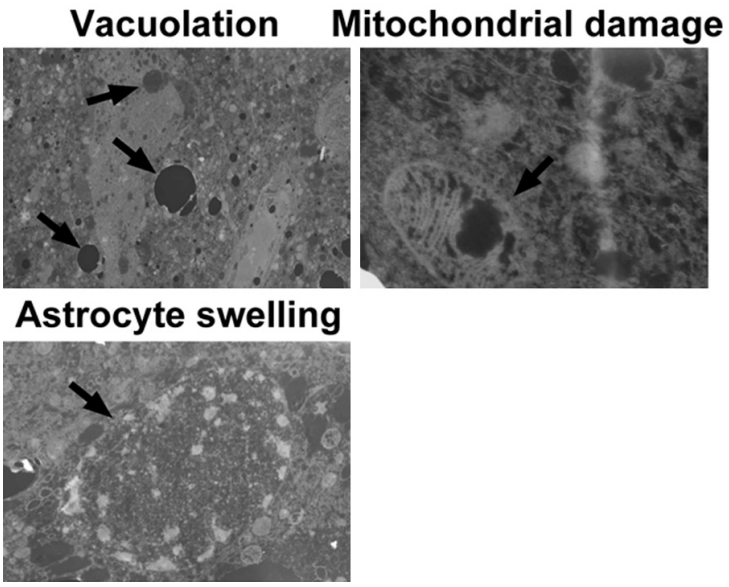

Figure 3. Ultrastructural changes in the epileptogenic zone derived from patients with refractory temporal lobe epilepsy. Upper left, neuronal degeneration, necrosis, and vacuolation formation are indicated by arrows. Magnification, × 2000. Upper right, mitochondrial swelling, vacuolation, and mitochondrial cristae fragmentation are indicated by an arrow. Magnification, × 20000. Lower left, astrocyte swelling, cytoplasmic hypervacuolization, condensation, and margination of nuclear chromatin are shown as indicated by an arrow. Magnification, × 6000.

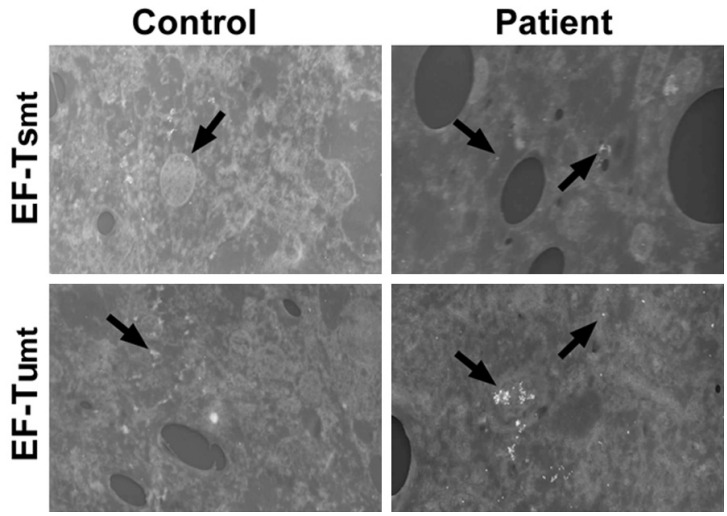

Figure 4. Immune electron microscopy analysis of the subcellular localization of EF-Tu$_{mt}$ and EF-Ts$_{mt}$ in brain tissues derived from control subjects and patients with refractory temporal lobe epilepsy. Samples were stained with anti-EF-Tu$_{mt}$ or anti-EF-Ts$_{mt}$ antibodies. Control samples were temporal lobe cortex tissues derived from non-epileptic patients with traumatic brain injury. Few gold particles were observed in cytoplasm and mitochondria; examples are indicated by an arrow. Experimental samples of epileptogenic zone derived from patients with refractory temporal lobe epilepsy. Accumulated gold particles were observed, particularly dense in the mitochondria; examples are indicated by arrows. Magnification, × 25000 for upper left; × 20000 for others.

model for hereditary temporal lobe epilepsy [14]. The elevated mitochondrial activity may indicate an enhanced demand for energy during neuronal activation caused by repeated vestibular stimulation [14].

Epilepsy has been proposed as a critical neurological sign in the early presentation of mitochondrial disorders, such as mitochondrial encephalopathies (ME) [15]. Topiramate, an antiepileptic drug, has been shown to prevent epilepsy in the rat and has been demonstrated to exert its protective functions through inhibition of the mitochondrial permeability transition pore [16]. In the current study, all of the patients recruited were diagnosed with refractory temporal lobe epilepsy and exhibited a long disease course with repeated seizures. Therefore, abnormal energy supply and consumption may occur in these patients. Indeed, ultrastructural damage was observed in the mitochondria of cells in the epileptogenic zone of patients with refractory temporal lobe epilepsy, and the damage included mitochondrial swelling and mitochondrial cristae fragmentation. These findings supported the likelihood of mitochondrial dysfunction in these patients.

EF-Tu$_{mt}$ and EF-Ts$_{mt}$ are two key regulators involved in the process of mitochondrial protein translation [4, 5]. In mammalian tissues, the EF-Tu$_{mt}$ and EF-Ts$_{mt}$ are highly expressed in cells undergoing high levels of growth and metabolism, such as metabolically active cardiomyocytes, and their activity depends on phosphorylation [17]. In addition, up-regulation of EF-Tu$_{mt}$ has been observed in tumors, further suggesting that the expression of mitochondrial elongation factors may be positively

Table 2. Difference in average numbers of EF-Tu_{mt}- or EF-Ts_{mt}-positive particles in brain tissues from epileptics and controls, as determined by immune electron microscopy

Group	Factor	Number of positive cells	*t*	*p* ($\bar{X}$ ± S)
Controls	EF-Tu_{mt}	32.67 ± 10.73	3.44	<0.01
Epileptics	EF-Tu_{mt}	112.76 ± 20.79		
Controls	EF-Ts_{mt}	33.50 ± 8.62	3.36	<0.01
Epileptics	EF-Ts_{mt}	111.48 ± 19.55		

related to mitochondrial function as well as active metabolism [18, 19].

Previous studies using the rat experimental chronic epilepsy model have demonstrated that the expression of EF-Tu_{mt} and EF-Ts_{mt} in cortex, hippocampus and striatum become gradually increased as a seizure attack is prolonged [20]. Furthermore, the up-regulation of EF-Tu_{mt} and EF-Ts_{mt} was shown to be accompanied by enhanced mitochondrial protein synthesis in rat brain [20]. In accordance with that rodent-based study, we detected a significantly up-regulated expression pattern for both EF-Tu_{mt} and EF-Ts_{mt} in human subjects with refractory temporal lobe epilepsy. Thus, we propose that, during a seizure attack, the protein translation process might be accelerated and the synthesis of mitochondrial respiratory enzymes might be greatly enhanced, leading to increased generation of ATP. This elevated energy supply may be necessary to meet the increased demand for energy that is required during neuronal activation caused by seizure stimulation. In addition, no significant difference was found in the expression of either EF-Tu_{mt} or EF-Ts_{mt}, which may reflect the fact that these two proteins form an EF-Tu·Ts complex that functionally contributes to mitochondrial protein synthesis [21].

Together with the findings from previous reports in the literature, our results suggest that abnormal activation of the mitochondrial translation process and the up-regulation of EF-Tu_{mt} and EF-Ts_{mt} are positively associated with the pathogenesis and progression of epilepsy. Our findings provide basic evidence for better understanding the pathogenesis and progression of epilepsy and offer a novel target for clinical therapy of epilepsy. However, the underlying mechanism of mitochondrial elongation factor-mediated mitochondrial activation and seizure incidence remains to be fully elucidated and further investigations are needed to understand the molecular mechanisms and electrophysiological process involved.

Disclosure of conflict of interest

None.

Authors' contribution

All authors were involved in data collection and integration. Lichao Sun and Qun Wang wrote the draft of the manuscript. Jiqing Qiu, Hongmei Song and Zhanpeng Zhu performed the epilepsy operations. Weihong Lin was responsible for the experiment concept, design and guidance of the study.

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