Research report

Correlation between tumor necrosis factor alpha mRNA and microRNA-155 expression in rat models and patients with temporal lobe epilepsy

Tao-Ran Li, Yan-Jie Jia, Qun Wang, Xiao-Qiu Shao, Ping Zhang, Rui-Juan Lv

1. Introduction

Epilepsy has an important influence on the health and psychosocial well-being of patients, and patients with epilepsy are more likely to suffer from abuse and violence (Nimmo-Smith et al., 2016). It is estimated that epilepsy affects at least 50 million people worldwide (Reynolds, 2002). The prevalence in developing countries (10–40‰) (de Bittencourt et al., 1996; Debrock et al., 2000; Preux and Druet-Cabanac, 2005) is higher than that in developed countries (4.9‰) (Ngugi et al., 2010), so it is probably under-estimated due to the limited professional and diagnostic resources in developing countries. Epilepsy constituted more than 0.5% of the global burden of disease in 2000 (Leonardi and Ustun, 2002), and Hong et al. reported that the average cost to each Chinese epilepsy patient per year was approximately ¥5253, more than half of their yearly income (Hong et al., 2009). Although the introduction of new anti-epilepsy drugs (AEDs) in recent decades has provided an improved range of options for epilepsy patients (Dichter and Brodie, 1996), however, it is estimated that 20% to 40% of epilepsy patients will develop drug resistant epilepsy (Liu et al., 2015). The failure of drug treatment may be caused by an incomplete understanding of the pathophysiological mechanism in drug refractory epilepsy. Thus, there exists an unmet need for newer antiepileptic drugs targeting newer targets with different mechanisms of action.

Temporal lobe epilepsy (TLE) is one of the most common pharmacoresistant focal epilepsies that usually accompany hippocampal sclerosis (HS) (Sendrowski and Sobaniec, 2013;
Chatzikonstantinou, 2014). Previous studies have noted that brain inflammation is an intrinsic feature of the hyperexcitable pathological brain tissue in pharmacoresistant epilepsy of different etiologies, and inflammation might be a consequence as well as a cause of epilepsy (Vezzani et al., 2011).

Tumor necrosis factor alpha (TNF-α) is an important inflammatory mediator involved in TLE. In rodent models of epilepsy, pharmacological or electrical stimulation of seizures triggers rapid induction of TNF-α in different regions of the brain (Minami et al., 1991; de Bock et al., 1996; De Simoni et al., 2000; Plata-Salaman et al., 2000; Dhote et al., 2007; Ashhab et al., 2013). Furthermore, increased TNF-α expression is found in the brain tissues of TLE patients compared with those of autopsy controls (Lachos et al., 2011; Varella et al., 2011; Ashhab et al., 2013; Teocchi et al., 2013). TNF-α also contributes to seizure activity and related pathology (Shandra et al., 2002; Balosso et al., 2005, 2009), and the pro-convulsive effect in the brain is mediated by the neuronal p55 receptor at low concentrations (Shinoda et al., 2003; Yuhas et al., 2003; Balosso et al., 2005; Stellwagen et al., 2005). Probert et al. found that the overexpression of brain TNF-α in transgenic mice was associated with the occurrence of sporadic spontaneous recurrent seizures (SRS) and chronic inflammation (Probert et al., 1997).

MicroRNAs (miRNAs) have emerged as important posttranscriptional regulators of gene expression during the past several years, providing a completely new level of controlling gene expression. miRNAs are short (20–23 nucleotides) non-coding RNAs that recognize partially complementary target sequences in selected mRNAs and predominantly inhibit protein expression by either destabilizing their mRNA targets or inhibiting protein translation (Ambros, 2004; Bartel, 2004; Kosik, 2006; Eulalio et al., 2008). The evidence demonstrates that miRNAs are important in the pathogenesis of inflammatory and immune processes (O’Connell et al., 2010). Recently, some studies have explored the critical role of miRNAs in TLE and suggested new therapeutic targets for the treatment of TLE (Alsharafi and Xiao, 2015; Alsharafi et al., 2015).

MicroRNA-155 (miR-155) is an inflammation-related miRNA and can mediate the acute inflammatory response associated with activation of Toll-like receptors, which is a suspected feature of epileptic onset (Tili et al., 2007; Maroso et al., 2011). Prior studies verified that regulating the expression of miR-155 in TLE rodent models can contribute to the alleviation of epilepsy (Cai et al., 2016; Huang et al., 2017). Furthermore, these studies also found that miR-155 was significantly up-regulated in the hippocampi obtained from TLE patients as well as in experimental TLE rats (Ashhab et al., 2013; Cai et al., 2016; Huang et al., 2017).

miR-155 has been identified and characterized as a component of the primary macrophage response to different types of inflammatory mediators (Taganov et al., 2006; O’Connell et al., 2007). Thus, TNF-α can stimulate the up-regulation of miR-155 in vitro (Tili et al., 2007; Stanczyk et al., 2008; Pedersen et al., 2009). Conversely, previous studies found that miR-155 can directly increase TNF-α levels by augmenting transcript stability through binding to its 3’UTR (Bala et al., 2011), and in vivo studies showed that miR-155 probably directly targets transcript coding for several proteins involved in lipopolysaccharide (LPS) signaling while enhancing TNF-α translation (Tili et al., 2007).

Only one previous study reported a strong relationship between miR-155 and TNF-α in immature TLE rat models and children with TLE (Ashhab et al., 2013). Furthermore, there were no studies from mature rodent models or adult TLE patient tissues. The aim of the present study was to detect the dynamic expression of TNF-α as a pro-inflammatory cytokine and miR-155 as a posttranscriptional inflammation-related miRNA in the hippocampi of mature rats following kainic acid (KA; Sigma Aldrich, USA)-induced status epilepticus (SE) during different phases of TLE development and in patients with TLE. This study was the first to explore the relationship between miR-155 and TNF-α by regulating the expression of miR-155 in TLE rat models.

2. Results

2.1. Behavior and seizures in rat models

After microinjecting KA into the CA3 region of the posterior hippocampus, the behavioral seizures of rats were continuously monitored for 24 h by video recording. Rat behavior was assessed by three experienced neurosurgical researchers who were unaware of the treatments given. Generally, within 2 h after waking from anesthesia, rats were characterized by continuous limbic seizure activity (stereotyped snifing, head nodding, gnawing, behavioral arrest, wet dog shakes, straub tail, decreased responsiveness), which was repeatedly interrupted by secondarily generalized convulsive seizures. This status lasted more than 40 min for all rats, and the rats showed a state of exhaustion after SE. In each KA-treated rat, clinical signs of seizure activity were observed. All rats exhibited a well-defined pattern of behavior following KA injection. Behavioral changes were consistent with the features of human TLE. All fifty rats in the experimental group successfully developed SE, and four rats died in the first 24 h. Saline control rats, which received injections of a comparable volume of saline in lieu of KA, did not exhibit any seizures, and no rats died in the first 24 h. Following administration, the rats were monitored for 2 months (15 h/day) using an infrared ray monitor and eye observation. The acute seizure and acute control groups were euthanized 2 h after SE. The remaining animals in the control group (n = 19) and the experimental group (n = 38) were kept individually in transparent Plexiglas cages (four rats per cage) to be used for further experiments. We also used the modified Racine scale (Racine, 1972) to observe SRS, which is widely accepted (Williams et al., 2009; Rattka et al., 2013). Racine Class I and II seizures were typically described as non-convulsive seizures, and they were hard to observe, while Racine class III, IV and V seizures were characterized as convulsive motor seizures. Only rats with this performance were considered for further research. Fourteen rats failed to develop SRS or died during the process, and they were excluded from the study. All control group rats survived after surgery, and no rats died during feeding post-surgery. The latent seizure and latent control groups were euthanized 7 days and 21 days post-SE without any behavioral changes or seizures. The remaining chronic seizure group rats manifested with chronic TLE, experiencing seizures with symptoms as described above. We observed SRS occurring mainly 5 weeks after SE. Chronic seizures occurred at 8 weeks, with a frequency of 2–10 seizures per day.

2.2. Dynamic expression pattern of TNF-α in rat models during the three phases of TLE development

To determine the dynamic expression pattern of TNF-α in the TLE rat models during the acute, latent and chronic phases, the rats were euthanized at the three phases post-SE separately. The hippocampus was collected immediately. TNF-α was detected using qRT-PCR. The results showed up-regulation of TNF-α expression in the rat hippocampal tissues in the epileptic groups during the three different phases of TLE development compared with the control groups (p < 0.01). Interestingly, the expression of TNF-α was highest in the acute phase (2 h post-SE) in the seizure and control groups. TNF-α expression was up-regulated in the CS group compared to the LS2 group (p < 0.01) when we compared these data horizontally. However, there was no significant difference between the CC group and LC2 group. In summary, TNF-α expression during
TLE development was gradually decreased in the latent phase post-SE but rose again in the chronic phase. In the rat hippocampal tissues, TNF-α expression was normalized to that of β-actin (Fig. 1A).

### 2.3. Relative expression of TNF-α in patients with TLE

To certify the expression pattern of TNF-α in the TLE rat models during the chronic phases, we examined TNF-α expression in patients with TLE. The results also showed significant up-regulation of TNF-α expression in the hippocampal tissues obtained from patients with TLE compared with the control patients (p < 0.01). In the patient hippocampal tissues, TNF-α expression was normalized to that of β-actin (Fig. 1B).

### 2.4. Dynamic expression pattern of miR-155 in rat models during the three phases of TLE development

To determine the dynamic expression pattern of miR-155 in TLE rat models during the acute, latent and chronic phases, we euthanized the rats at the three phases post-SE separately. The hippocampus was collected immediately. miR-155 was detected using qRT-PCR. The results showed significant up-regulation of miR-155 expression in the epileptic groups compared with the control groups during the latent and chronic phases of TLE development (p < 0.01). Expression was lower in the latent phase 2 (LS2 group) compared with that in the latent phase 1 (LS1 group) and showed the lowest expression in the chronic phase (CS group). Notably, there was no difference between the AS group and AC group, which suggested that miR-155 expression was not regulated in the acute phase. In summary, miR-155 expression started to increase 2 h post-SE and reached a peak level 7 days post-SE, then it gradually decreased. In the rat hippocampal tissues, miR-155 expression was normalized to that of the U6 small nuclear RNA gene (U6; Fig. 2A).

### 2.5. Relative expression of miR-155 in patients with TLE

To certify the expression pattern of miR-155 in TLE rat models during chronic phases, we examined miR-155 expression in patients with TLE. The results showed significant up-regulation of miR-155 expression in the hippocampal tissues of TLE patients compared with controls (p < 0.01). In the patient hippocampal tissues, miR-155 expression was normalized to that of U6 (Fig. 2B).

### 2.6. Effect of miR-155 antagomir on the miR-155 expression level

To validate the effect of miR-155 antagomir on miR-155 expression in TLE rat models, we examined hippocampal miR-155 expression using the miR-155 antagomir or antagomir-control. In the LS1 antagonism group or LS1 control group, rats received antagomir or antagomir-control respectively, 1 h after SE, and the rats were euthanized 7 days post-SE. The results showed no significant difference in miR-155 expression between the LS1 control group and LS1 group, which suggested that the miR-155 antagomir-control had no effect on miR-155 expression. Furthermore, the expression of miR-155 in the LS1 antagonism group was down-regulated compared to that in the LS1 control group or LS1 group (p < 0.01), which suggested that the antagonist was effective. miR-155 expression was normalized to that of U6 (Fig. 3).

### 2.7. Effect of miR-155 antagomir on TNF-α expression level

To determine the effect of the miR-155 antagonist on TNF-α expression in TLE rat models, we examined hippocampal TNF-α expression using the miR-155 antagonist or antagonism-control. The results showed there was no significant difference in TNF-α expression between the LS1 control group and LS1 group, which suggested that the miR-155 antagonist-control had no effect on TNF-α expression. Furthermore, the expression of TNF-α in the LS1 antagonist group was down-regulated compared to that in the LS1 control group or LS1 group (p < 0.01; p < 0.05), which suggested that the miR-155 antagonist can inhibit TNF-α expression. TNF-α expression was normalized to that of β-actin (Fig. 4).

### 3. Discussion

Recently, several lines of research have provided strong support for the hypothesis that inflammatory processes within the brain...
might constitute a common and crucial mechanism in the pathophysiology of seizures and epilepsy in experimental models and patients (Vezzani and Granata, 2005; Lee et al., 2007; Vezzani and Baram, 2007; Choi et al., 2009; Auvin et al., 2010; Riazi et al., 2010; Vezzani et al., 2011, 2013). Furthermore, steroids and anti-inflammatory treatments display anticonvulsant activity in some drug-resistant epilepsy patients (Riikonen, 2004; Verhelst et al., 2005; Wirrell et al., 2005; Wehess et al., 2007; Suleiman and Dale, 2015) and animal models (Vezzani et al., 2000; Jung et al., 2006; Marchi et al., 2011; Maroso et al., 2011). Recent studies have shown that TNF-α is up-regulated in the hippocampal tissues of TLE animal models (Minami et al., 1991; de Bock et al., 1996; De Simoni et al., 2000; Plata-Salaman et al., 2000; Turrin and Rivest, 2007).
TNF-α observed that TNF-α is equivalent to those in the chronic phase of the rat models. We (Minami et al., 1991; Plata-Salamán et al., 2000). However, was up-regulated 2 h after SE in different epilepsy models (Ashhab et al., 2011; Dhote et al., 2007; Ashhab et al., 2013), which was verified in patients with TLE (Lachos et al., 2011; Varella et al., 2011; Ashhab et al., 2013; Teocchi et al., 2013). An improved clear understanding of those molecules and of the functional mechanisms that regulate the expression of this pro-inflammatory cytokine may offer new therapeutic targets for the treatment of TLE. In recent years, miRNAs have been found to play a crucial role in regulating the gene expression of a considerable part of the human genome in neurons, where miRNAs are expressed at all stages of development and involved in neuronal differentiation, synaptogenesis, and plasticity (Schratt, 2009). The involvement of miRNAs in neurological diseases has been highlighted: the levels of several miRNAs are altered chronically in response to brain injuries such as surgeries, ischemic strokes, brain hemorrhage or prolonged seizures (Liu et al., 2010; Hu et al., 2011). Emerging evidence indicates the participation of miRNAs in lesions of all types of epilepsy (Li et al., 2014). Importantly, these studies reveal a tight correlation between miRNA deregulation and neuroinflammation, seizure-induced neuronal death and other related biological pathways. It is clear that miRNAs regulate a wide variety of pathogenic and functional mechanisms during TLE development.

Previous studies indicated that the deregulation of miR-155 was associated with different kinds of cancer, cardiovascular diseases and viral infections (Farooqi et al., 2009). It is also inextricably linked with inflammation (Taganov et al., 2006; Stanczyk et al., 2008) and able to mediate the acute inflammatory response associated with the activation of Toll-like receptors, which is a suspected feature of epilepsy onset (Tili et al., 2007; Maroso et al., 2011). Thus, inflammation seems to highlight a potential connection between miR-155 and epilepsy, where the up-regulation of miR-155 might play an important role. Several reports have shown that the level of miR-155 is significantly up-regulated in TLE rodent animals (Ashhab et al., 2013; Cai et al., 2016; Huang et al., 2017), which supports the conclusion that the expression of miR-155 is abnormally high in TLE patients (Ashhab et al., 2013; Huang et al., 2017). It was also found that inhibition of miR-155 with specific antagonists results in the alleviation of seizure behavior and protects the hippocampus from apoptosis (Cai et al., 2016; Huang et al., 2017). However, due to limited data availability, more in depth research in this area is needed to explain how miR-155 has its effects.

In the present study, we demonstrated an association between TNF-α as a pro-inflammatory cytokine and miR-155 as a posttranscriptional inflammation-related miRNA during the three phases of TLE development in rat models. We confirmed our results by examining their expression levels in patients with TLE, which were equivalent to those in the chronic phase of the rat models. We observed that TNF-α secretion and the miR-155 expression levels were correlated after SE and that their expression was higher than control groups during the latent and chronic phases of TLE development. In addition, TNF-α expression was inhibited when we suppressed miR-155 expression with specific antagonists, suggesting that miR-155 could regulate brain inflammation by regulating TNF-α. These revealed their underlying functions during the process of TLE development.

In the acute phase, TNF-α was excessively high in both the TLE and control groups, and the levels of the former (AS group) were higher than those of the latter (AC group). However, no significant dysregulation of miR-155 was detected in the acute phase. In line with our observation, two previous studies also found that TNF-α was up-regulated 2 h after SE in different epilepsy models (Minami et al., 1991; Plata-Salamán et al., 2000). However, Dhote et al. established epilepsy models by soman and observed the up-regulation of TNF-α postponed to 6 h post-SE (Dhote et al., 2007). In addition, Ashhab et al. found that the expression of TNF-α in control groups had no huge fluctuations (Ashhab et al., 2013). The results of those two studies were inconsistent with our study. A possible explanation for this might be that we established TLE rat models by injecting KA into the hippocampus, which differed greatly with the systemic administration of soman or pilocarpine (Lévesque and Avoli, 2013), and the operation was a trauma that could result in the release of TNF-α. Even so, TNF-α expression was significantly up-regulated in the AS group compared with the AC group. For miR-155 in the acute phase, Ashhab et al. found that the expression of miR-155 was up-regulated in the TLE group 2 h post-SE, which was different from our study (Ashhab et al., 2013). A recent study performed by Huang et al. found that miR-155 expression was not regulated until 1 day post-SE (Huang et al., 2017). Cai et al. measured the expression of miR-155 in the TLE group 4 h and 12 h post-SE, and found that the expression was up-regulated at both time points compared to that of the control group; however, expression at the latter time point was higher than that at the former, which suggested to the researchers that the expression of miR-155 was time-dependent post-SE (Cai et al., 2016). These two recent studies support our results indirectly since the expression of miR-155 increased gradually and was not regulated 2 h post-SE (Cai et al., 2016; Huang et al., 2017). Furthermore, these findings indicated that the up-regulation of miR-155 lagged behind TNF-α, which was consistent with our results.

In the latent phase 1 (7 days post-SE), TNF-α expression was obviously decreased compared with the acute phase, but the LS1 group was still higher than the LC1 group. There were no significant differences among the LC1, LC2 and CC groups, which suggested that TNF-α expression in the control groups was stable. miR-155 showed significant up-regulation in the LS1 group, with a peak level compared with AS, LS2 or CS groups. In latent phase 2 (21 days post-SE), TNF-α expression was the lowest during three phases of TLE group, but it was still higher than that of the control group. miR-155 expression in the LS2 group was lower than that in the LS1 group, but it was also higher than that in the LC2 group. In this seizure-free phase (LS1 and LS2), pathophysiologic changes may occur in preparing the imminent epilepsy and up-regulation of TNF-α expression in TLE groups can still be observed; however, TNF-α expression gradually decreased compared with the acute phase. Bock et al. measured the expression of TNF-α in a seizure group 2 days and 7 days post-SE, and they found TNF-α expression was up-regulated compared to the corresponding control group (de Bock et al., 1996). However, the expression of TNF-α in seizure group 7 days post-SE was not obviously different from the expression of TNF-α 2 days post-SE (de Bock et al., 1996). Our study found that TNF-α expression was up-regulated in the LS1 group compared to the LC1 group, which was similar to the study of Bock et al. (de Bock et al., 1996). Furthermore, the expression of TNF-α reached its peak level 2 h post-SE and then gradually decreased until 21 days post-SE (LS2 group). The peak value of TNF-α at 2 h post-SE was partially because of the operation, and TNF-α expression may decrease obviously during the period of 2 h to 2 days. However, we did not examine the expression at 2 days post-SE. If we examined the expression at 2 days post-SE, we may observe a similar result that the expression at 2 days post-SE was similar to the expression at 7 days post-SE. Furthermore, the similar results obtained 3 or 4 days post-SE indirectly support our data 7 days post-SE (Yang et al., 2016; Sedaghat et al., 2017). Plata-Salamán et al. analyzed the expression of TNF-α in multiple regions of amygdala kindling rats and found that it was not changed 21 days post-SE (Plata-Salamán et al., 2000). Similar results were reported by Ashhab et al., who found that the expression of TNF-α was elevated but not statistically changed in pilocarpine intraperitoneally induced rats (Ashhab et al., 2013). We speculated that these inconsistencies maybe due to the different modeling methods, and the effect of chemical drugs on the hippocampus was greater than that.
of physical stimulation or the method of intraperitoneal injection. miR-155 reached a peak level at 7 days post-SE, which lagged compared with TNF-α in our study. The result was consistent with Huang et al.’s study, which found that the peak value was delayed to 14 days post-SE compared with 1 day, 30 days and 60 days post-SE, and the peak time occurred during the latent phase of TLE development (Huang et al., 2017).

In the chronic phase (60 days post-SE), the expression levels of both TNF-α and miR-155 in the CS group were higher than those in the CC group. Consistent with the chronic phase findings in rat models, we found up-regulation of TNF-α and miR-155 in patients with TLE. Previous studies also reported the up-regulation of miR-155 (Ashhab et al., 2013; Huang et al., 2017) and TNF-α (Lachos et al., 2011; Varella et al., 2011; Ashhab et al., 2013; Teocchi et al., 2013) in TLE patients. The expression of TNF-α gradually decreased after SE and reached the lowest level in latent phase 2, which meant that inflammation might be suppressed in the seizure-free phase. However, its expression in the CS group was higher than that in the LS2 group, which suggested that it might promote SRS. The expression of miR-155 reached its peak level at 7 days post-SE and then gradually decreased until 60 days post-SE, which was consistent with Huang et al.’s study (Huang et al., 2017). In their study, miR-155 expression reached peak levels at 14 days post-SE and then gradually decreased until 60 days post-SE. Based on previous results, the current study suggested that the variation trend of miR-155 lagged behind TNF-α, so perhaps miR-155 started to rise after 60 days post-SE. Combined with the results of the study performed by Ashhab et al. (2013), these results suggested that TNF-α and miR-155 had mutual effects.

Evidence is emerging that inflammation is not just a cause of epilepsy but is also a consequence (Vezzani et al., 2011). Several inflammatory mediators have been detected in surgically resected brain tissue from patients with refractory epilepsies (Vezzani and Granata, 2005; Ravizza et al., 2008; Kan et al., 2012; Omar et al., 2012; Ashhab et al., 2013; Fiala et al., 2013). Furthermore, mounting evidence for a role in inflammatory processes in experimental TLE rodent models have identified the fact that putative triggers of brain inflammation in epilepsy, and provided mechanistic insights into the reciprocal causal links between inflammation and seizures (Choi et al., 2009; Riazi et al., 2010; Li et al., 2018).

The above studies have shown that seizure activity per se can induce brain inflammation, where recurrent seizures initiate a cascade of inflammation and then lead to the perpetuate inflammation of epilepsy. Drugs aimed at this vicious cycle may be new targets for the treatment of TLE in the future.

In this study, we established TLE rat models by injecting KA into the hippocampus, which can reflect the neuropathological and electroencephalographic features that were seen in patients with TLE (Lévesque and Avoli, 2013). We monitored these rats continuously until 60 days post-SE and obtained hippocampi at three particular phases of TLE development. After detecting TNF-α and miR-155 using qRT-PCR, we found that the two molecules were in roughly a parallel relationship. Since the specific connections required further experiments, we explored the effect on TNF-α expression levels by antagonizing miR-155. After inhibiting miR-155 expression levels using the miR-155 antagonist in TLE rat models, we found that TNF-α expression can also be inhibited. This study was different from the study performed by Ashhab et al. (2013). First, our study confirmed how modifying miR-155 influences TNF-α expression. In contrast, they observed the dynamic changes in miR-155 by regulation of TNF-α expression at the cell level. Second, the TLE disease state in young and adult humans and rats were different. Immature rats were relatively resistant to neuronal loss and synaptic changes in TLE animal models (Lado et al., 2000), resulting in great differences in their susceptibility to SRS and the ability to spread from the hippocampus to other parts of the brain (Tsukamoto et al., 1991). Third, different drugs and ways of administration were used, which also might lead to inconsistent results (Chung et al., 2015). miR-155 can directly affect TNF-α according to bioinformatics predictions. In addition, previous studies found that miR-155 affected TNF-α mRNA stability because miR-155 inhibition decreased, whereas miR-155 overexpression increased, TNF-α mRNA half-life (Bala et al., 2011). In vivo studies showed that transgenic mice overexpressing miR-155 in a B-cell lineage produce more TNF-α when challenged with LPS, and miR-155 most likely directly targets transcript coding for several proteins involved in LPS signaling while enhancing TNF-α translation (Tili et al., 2007). Therefore, we did not explore the exact mechanism by which miR-155 influences TNF-α levels in this study.

In conclusion, the current study shows that TNF-α and miR-155 are involved in the pathogenesis of TLE development and that their parallel expression patterns during the phases of TLE development show an interactive relationship between the two markers, suggesting that miR-155 might play a critical role in the regulation of the neuroinflammatory response during the pathogenesis of TLE. We are the first to examine the relationship between miR-155 and TNF-α expression by regulating the expression of miR-155 in TLE rat models. Our findings support the idea that the miR-155/TNF-α axis may be a novel therapeutic target for TLE.

4. Materials and methods

The authors declare that all methods were performed in accordance with the relevant guidelines and regulations.

4.1. Animals

Specific pathogen-free (SPF) adult male Sprague-Dawley (SD) rats (250–280 g, 7 weeks) were obtained from Beijing Vital River Laboratory Animal Technology Company Limited (Beijing, China) and kept under SPF conditions. All animal studies were performed according to the guidelines of the Guidance for Animal Experimentation of Capital Medical University, and the protocol was approved by the Experimental Animal Ethics Committee of Beijing Tiantan Hospital, Capital Medical University (Permit No. 201502002). All surgeries were performed under chloral hydrate anesthesia.

4.2. Experimental seizure models

The 75 adult male SD rats were randomly divided into two groups: experimental group (n = 50) and control group (n = 25). All rats (250–280 g) were anesthetized with 10% chloral hydrate (0.3 ml/kg) intraperitoneally and then mounted in a stereotaxic frame (David Kopf Instruments, USA). Then, the experimental group rats were operated on according to the following procedure: 0.8 μg (1 μg/μl in saline) KA was injected in the CA3 region of the left hippocampus with the following coordinates (on the basis of the rat brain atlas of Paxinos and Watson, the Rat Brain in Stereotaxic Coordinates, Sixth Edition): anterior posterior (AP), –5.64 mm; mediolateral (ML), 4.7 mm; dorsoventral (DV), 6.8 mm, relative to bregma. The injection was conducted with a 1.0-μl microsyringe (RWD Life Science & Technology, China) and persisted for 5–10 min to avoid leaking of the solution. Control group rats received an injection of the same amount of normal saline as a replacement for KA.

4.3. Video monitoring of seizures

After surgery, all rats were continuously monitored for behavioral seizures in the first 24 h with video recording in the cages.
The severity of behavioral seizures was scored according to the modified Racine scale (Racine, 1972) as follows: I, mouth and facial movements; II, head nodding; III, forelimb clonus and a lordotic posture; IV, rearing with forelimb clonus; and V, rearing, forelimb clonus and falling. SE was defined as the onset of continuous generalized (score 4–5) seizure activity lasting no less than 40 min, and only those animals that reached SE were included in this study. The KA-induced SE was not terminated pharmacologically, and no special care was given to the individuals.

The rats that survived from the acute phase were raised in cages with adequate water and food. Starting from the first 24 h, the rats received video monitoring 13 h/day (19:00–08:00) and were observed in the afternoon (13:00–19:00). The video recording lasted until 60 days post-SE.

Based on epilepsy development phases, the experimental and control groups of rats were each randomly divided into four groups as follows: (1) acute control group, AC (control rats, 2 h after saline administration, n = 6); (2) acute seizure group, AS (SE rats, 2 h after KA administration, n = 8); (3) latent control group1, LC1 (control rats, 7 days after saline administration, n = 6); (4) latent seizure group1, LS1 (SE rats, 7 days after KA administration, n = 8); (5) latent control group2, LC2 (control rats, 21 days after saline administration, n = 6); (6) latent seizure group2, LS2 (SE rats, 21 days after KA administration, n = 8); (7) chronic control group, CC (control rats, 60 days after saline administration, n = 6) and (8) chronic seizure group, CS (SE rats, 60 days after KA administration, n = 8).

4.4. miR-155 antagonim experiments

An extra 18 adult male SD rats were used in the miR-155 antagonim experiments. Briefly, all rats received 0.8 μg KA injections as previously described. After surgery, they all exhibited continuous generalized seizure activity lasting no less than 40 min. miR-155 expression in the rat hippocampus following SE was antagonized using an antagonim that specifically and efficiently targets miR-155. A miR-155 antagonim or an antagonim-control (Ribo-Bio Co., Ltd., China) was dissolved in normal saline at a concentration of 1 nmol/10 μl. Eighteen rats were randomly divided into 2 groups as follows: latent antagonim group (LS1 antagonim, n = 9) and latent antagonim-control group (LS1 control, n = 9). One h after SE, rats received antagonim or antagonim-control (1 nmol for each rat) infused at a very slow rate (more than 10 min and persisted for 5–10 min) by microsyringe into the lateral ventricle (with the following coordinates: AP = −0.84 mm; ML = 1.8 mm; DV = 3.5 mm, relative to bregma). At the seventh day after SE onset, rats were euthanized and hippocampal tissues were quickly removed for detection of miR-155 and TNF-α.

4.5. TLE patients and controls

This study was approved by the Institutional Ethics Committee of Beijing Tiantan Hospital conforming to the principles expressed in the Declaration of Helsinki, and written informed consent was obtained from all patients and control participants. Tissue samples were obtained at surgery from seven patients with unilateral drug refractory TLE, and typical imaging features and pathological confirmation of HS (four left and three right) who had unilateral selective amygdalohippocampectomy (Table 1). Surgical samples were subjected to routine histopathological examination. Eight normal hippocampal samples as controls were obtained from patients with no history of epilepsy (gifted from Peking Union Medical College Hospital) (Table 2). All autopsies were performed within 8 h of death. Neuropathologic examination confirmed that the control tissues were normal. Clinical information for patients with TLE and controls is shown in Table 1.

4.6. Rat tissue preparation and RNA isolation

Rats were euthanized at 2 h, 7, 21 and 60 days post-SE or saline administration under deep anesthesia using 10% chloral hydrate (5 ml/kg i.p.). After decapitation, hippocampus was quickly removed from the brain; they were rapidly cooled in liquid nitrogen more than 30 min and stored at −80 °C until use. Each group of animals was processed independently, and the whole process was carried out in iced saline to avoid RNA degradation and cross-contamination.

The total hippocampal RNA was isolated according to the manufacturer’s protocol (TRIzol reagent, Invitrogen, USA). RNA quality and quantity were determined at 260/280 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, ThermoScientific, USA).

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<th>Table 1</th>
<th>Clinical information on the patients with TLE.</th>
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<td>F</td>
</tr>
<tr>
<td>Patient 4</td>
<td>M</td>
</tr>
<tr>
<td>Patient 5</td>
<td>F</td>
</tr>
<tr>
<td>Patient 6</td>
<td>F</td>
</tr>
<tr>
<td>Patient 7</td>
<td>F</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ID</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Side of hippocampus sample</th>
<th>Causes of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>M</td>
<td>26</td>
<td>Left</td>
<td>Schizophrenia (commit suicide by jumping off a high building)</td>
</tr>
<tr>
<td>Control 2</td>
<td>F</td>
<td>44</td>
<td>Left</td>
<td>Mammary cancer</td>
</tr>
<tr>
<td>Control 3</td>
<td>F</td>
<td>16</td>
<td>Left</td>
<td>Leukaemia</td>
</tr>
<tr>
<td>Control 4</td>
<td>F</td>
<td>46</td>
<td>Left</td>
<td>Cervical carcinoma</td>
</tr>
<tr>
<td>Control 5</td>
<td>M</td>
<td>42</td>
<td>Right</td>
<td>Rectal cancer</td>
</tr>
<tr>
<td>Control 6</td>
<td>M</td>
<td>31</td>
<td>Right</td>
<td>Cerebral infarction</td>
</tr>
<tr>
<td>Control 7</td>
<td>F</td>
<td>22</td>
<td>Right</td>
<td>Heart disease</td>
</tr>
<tr>
<td>Control 8</td>
<td>F</td>
<td>48</td>
<td>Right</td>
<td>Choriocarcinoma</td>
</tr>
</tbody>
</table>

Abbreviations: TLE, temporal lobe epilepsy; TPM, topiramate; CZP, clonazepam; VPA, valproate; PB, phenobarbitalone; CBZ, carbamazepine; OXC, oxcarbazepine; LTG, lamotrigine; PHT, phenytoin; LEV, levetiracetam.
Table 3
Oligonucleotides used for qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD rats TNF-α</td>
<td>Forward</td>
<td>TGGCGTGTTCATCGGTTCCTACCC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCCCGAATCCAGCCACACTATT</td>
</tr>
<tr>
<td>SD rats β-actin</td>
<td>Forward</td>
<td>GCAACACACTGCTGTCGCTCT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TACTCTGCTGGTCTGTATACCA</td>
</tr>
<tr>
<td>SD rats miR-155</td>
<td>Forward</td>
<td>Synthesized by GeneCopoeia™</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>The reverse was the universal adaptor PCR primer in the kit</td>
</tr>
<tr>
<td>SD rats U6</td>
<td>Forward</td>
<td>CGCCTATGCTCAAGGCTTCTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>The reverse was the universal adaptor PCR primer in the kit</td>
</tr>
<tr>
<td>Human TNF-α</td>
<td>Forward</td>
<td>CTCTCTCTCCTGATGCTGG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCCTGTATCTCAGCTCCCTA</td>
</tr>
<tr>
<td>Human β-actin</td>
<td>Forward</td>
<td>CGGGACACATGGGAGAAAA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AAGGAAGGGCTGAAAGCTG</td>
</tr>
<tr>
<td>Human miR-155</td>
<td>Forward</td>
<td>Synthesized by GeneCopoeia™</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>The reverse was the universal adaptor PCR primer in the kit</td>
</tr>
<tr>
<td>Human U6</td>
<td>Forward</td>
<td>Synthesized by GeneCopoeia™</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>The reverse was the universal adaptor PCR primer in the kit</td>
</tr>
</tbody>
</table>

4.7. TNF-α or miR-155 expression by qRT-PCR in the hippocampi of rats and TLE patients

For TNF-α and β-actin, 2.5 μg of total RNA was reverse-transcribed into cDNA using oligo (dT) primers according to the GoScript Reverse Transcription System (A5001, Promega, USA). For miR-155 and U6, 4.0 μg of total RNA was reverse-transcribed into cDNA by polyA polymerase according to the manufacturer’s instructions (All-in-one™ miRNA qRT-PCR Detection Kit, GeneCopoeia™, USA).

The oligonucleotides used for qRT-PCR were synthesized for amplifying cDNA in SD rats and human samples are shown in Table 3. β-actin or U6 were used as the housekeeping gene. For TNF-α and β-actin, the master mix was prepared on ice containing 1 μl cDNA (diluted five times with RNase-free water), 10 μl of 2 × SuperReal PreMix Plus (FP205, TianGen Biotech, China), and 0.3 μM of both forward and reverse primers in each sample. The final volume was adjusted to 20 μl with RNase-free water. For miR-155 and U6, each sample contained 2 μl cDNA (diluted five times with RNase-free water), 10 μl mix (All-in-one™ miRNA qRT-PCR Detection Kit, GeneCopoeia™, USA), and 0.2 μM of both forward and reverse primers. The final volume was adjusted to 20 μl with RNase-free water. We evaluated every sample in triplicate to correct operating errors. The reaction conditions were set as follows: initial denaturation at 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 58 °C for 20 s and extension at 72 °C for 10 s. The fluorescent product was measured by a single acquisition mode at 72 °C after each cycle. A melting curve was obtained as mentioned above. Data were quantified using a modification of the 2^–ΔΔCt method as described previously (Livak and Schmittgen, 2001).

4.8. Statistical analysis

Statistical analyses were performed with SPSS 13.0 software (SPSS, Chicago, IL, USA). All data were expressed as the means ± s.d. Differences were evaluated by ANOVA. P < 0.05 was considered to be statistically significant.

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Competing interests

The authors declare that they have no conflicts of interest.

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Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

Tao-Ran Li performed all the experiments, prepared the figures and wrote the manuscript. Yan-Jie Jia, Qun Wang, Xiao-Qiu Shao and Ping Zhang contributed with scientific support. Rui-Juan Lv designed and supervised the experiments, coordinated the project and corrected the manuscript. All authors reviewed the manuscript.

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