



RESEARCH ARTICLE

HCMV infection and IFITM3 rs12252 are associated with Rasmussen's encephalitis disease progression

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Funding Information

This work was supported by Grants from the National Natural Science Foundation of China (No.81701992).

Received: 29 June 2020; Revised: 25 November 2020; Accepted: 1 December 2020

Annals of Clinical and Translational Neurology 2021; 8(3): 558–570

doi: 10.1002/acn3.51289

Abstract

Objective: Rasmussen's encephalitis (RE) is a rare and severe progressive epileptic syndrome with unknown etiology. Infection by viruses such as human cytomegalovirus (HCMV) has been hypothesized to be a potential trigger for RE. Interferon-induced transmembrane protein-3 (IFITM3) single-nucleotide polymorphism (SNP) rs12252 is associated with the severity of viral infection disease. This study aimed to address the possibility that HCMV infection and IFITM3 rs12252 might be associated with RE disease progression. **Methods:** The expression of HCMV and IFITM3 was detected with immunohistochemical staining, in situ hybridization and immunofluorescence double staining. The genotype of IFITM3 rs12252 was detected using the Sanger sequencing method. A genetic association analysis was carried out for this SNP and HCMV antigen expression. The relationship between this SNP and the clinical characteristics of these patients was further analyzed. In in vitro study, HCMV replication in SH-SY5Y cells with overexpressed IFITM3 variant was detected by immunofluorescence and real-time RT-PCR. **Results:** Elevated expression of HCMV and IFITM3 was observed in the brain tissue of RE patients. Moreover, the IFITM3 polymorphism rs12252-C was found to associate with HCMV high detection and rapid disease progression in RE patients with the IFITM3 rs12252-CC genotype. In vitro study showed the overexpressed IFITM3 variant was associated with HCMV high infection level. **Conclusion:** These results suggest that the IFITM3 rs12252-C is associated with the disease progression of RE patients via facilitating persistent HCMV infection in brain tissue and provides new insight into understanding the pathogenesis of RE.

Introduction

Rasmussen's encephalitis (RE) is a rare and severe progressive epileptic syndrome^{1,2} that was first reported by the Canadian neurosurgeon Theodore Rasmussen and his colleagues in 1958. The incidence of RE is approximately 1.8 per 10 million in Europe. The typical clinical manifestation is frequent seizures of partial epilepsy or status epilepticus accompanied by progressive unilateral hemiplegia and cognitive impairment. Hemispherectomy is the only effective treatment, resulting in 70%–80% seizure freedom. However, this treatment also leads to a severe decline in cognitive function and fine motor function of

limbs. Therefore, RE is highly detrimental to patients and their families.

The etiology and pathogenesis of RE remain controversial.² Because the histopathological features, including progressive neuronal loss, glial nodules, and perivascular cuffing in the RE brain, were known to be similar to those of other types of viral encephalitis, Rasmussen proposed viral infection as a possible cause.³ In addition, the fact that approximately half of RE patients had a history of viral infection and that few patients had a vaccination history further supported the viral infection theory.⁴ In the following several decades, the detection of a variety of viral antigens and nucleic acids in RE brain tissue was

reported, including Epstein–Barr virus (EBV), human cytomegalovirus (HCMV), herpes simplex virus (HSV-1), human papillomavirus (HPV), and enterovirus.^{5–10} Due to the lack of specificity of the virus detected, it was difficult to establish a causal relationship between viral infection and RE. However, based on obvious lymphocyte infiltration in RE brain tissue, it was speculated that viruses may serve as the triggering factor that could cause nervous system inflammation through interaction with the host immune system and then lead to chronic brain damage.

Human herpes viruses (HHVs) belong to *Herpesviridae*, a large family of double-stranded DNA viruses that consists of eight members, namely, HHV1–8. Latent and recurring infections are typical characteristics of this group of viruses. HCMV (HHV5) is a beta herpesvirus with neurotropism. Even healthy individuals are generally susceptible to HCMV, and its infection rate is as high as 70–100%. Infections with HCMV are also a major cause of morbidity and mortality in congenitally infected children and immunosuppressed individuals.¹¹ The incidence of congenital HCMV transmission ranges from 0.5% to 0.7% of pregnancies in the United States and other developed nations and up to 2.0% of pregnancies in the developing world.^{11,12} Approximately 13.5% of congenitally infected newborns are symptomatic.¹³ Congenital HCMV infection is the most common infectious cause of brain damage and hearing loss and occasionally causes death in newborns.¹⁴

The innate immune system of the host is the first line of defense against virus infection. As an important element of the innate immune system, interferon (IFN) plays a key role in the early stage of virus infection by activating the transcription of several IFN-stimulated genes (ISGs). The products of those ISGs take on a number of diverse roles; collectively, they are highly effective at controlling viral infection. Of those genes, the only ISGs that have been shown to have a bona fide role in blocking virus entry are members of the IFN-inducible transmembrane (IFITM) family. In humans, this family is composed of five members: IFITMs 1, 2, 3, 5, and 10. Little is known about the function of IFITM10. IFITM5 is expressed strictly in osteoblasts and is involved in bone mineralization and maturation. IFITM5 mutation was reported to be related to osteogenesis imperfecta type V. Recently, IFITMs 1, 2, and 3 were shown to be potent inhibitors of influenza A virus, Ebola virus, dengue virus, and Zika virus by interfering with the membrane fusion process of viruses or inhibiting the fusion of viral capsid and lysosomal membrane.^{15–17}

IFITM3 was first discovered in the early 1990s and was neglected until 2013, when Zhang et al. reported that a close relationship was found between the single-

nucleotide polymorphism (SNP) mutation rs12252-C of IFITM3 and severe influenza.¹⁸ The study demonstrated that the first 21 amino acids at the N terminus of IFITM3 inhibit virus uncoating by interacting with the lysosomal membrane to play an antiviral role. It is known that the rs12252-C mutation produces IFITM3 protein truncated by 21 amino acids at the N terminal (IFITM3 NΔ21). In this process, the physical and chemical properties of IFITM3 NΔ21 are changed considerably. The mutated protein is not flexible enough and does not effectively prevent fusion of the virus with the endocytic membrane, which, in turn, reduces the ability of the immune system to fight off virus infections. Therefore, the frequency of the IFITM3 rs12252-C allele and CC genotype is closely related to the severity of some viral infectious diseases.^{19–21} The frequency of IFITM3 rs12252 CC varies considerably among different populations and is rare in Caucasians. For example, the frequency of IFITM3 rs12252 CC is only 2% and 8% in France and Northern Europe, respectively. However, in China, approximately 25% of Han Chinese carries the IFITM3 rs12252 CC genotype (<http://www.1000genomes.org/>). It has also been reported that this genotype of the Han population in China is closely related to the rapid clinical progression of severe influenza and HIV infection.^{18,22}

Previously, we found elevated HCMV expression in the brain tissue of RE patients with an 88.5% positive rate.²³ IFITM3 was able to effectively inhibit HCMV infection in vitro and in vivo.^{24,25} Therefore, it is speculated that the IFITM3 rs12252 CC genotype is involved in high-HCMV detection and then participates in RE pathogenesis. In this study, we determined the association between the IFITM3 rs12252 variant and the pathogenesis of RE by examining RE cases.

Materials and Methods

Patients

Forty-five RE patients who were admitted to Sanbo Brain Hospital from April 2008 to December 2017 were included in the study. The clinical diagnosis was made according to the European diagnosis criteria.¹ This study was approved by the Ethics Committee of Sanbo Brain Hospital, Capital Medical University (2013061801), and written informed consent was obtained from all participants or their guardians prior to the study. After craniotomy, at least two blocks of brain tissue from the same area were collected from the RE patients. One block was fixed, embedded in paraffin and sliced into 6 μm thickness and then used for pathohistological, immunohistochemical, and double immunofluorescent staining. The other block was immediately snap-frozen after the

resection and then used for the detection of cytokines. Control brain samples were obtained from brain autopsies of 20 patients with no history of neurological disease. Forty-two patients who were diagnosed with temporal lobe epilepsy (TLE) were included as controls in the sequencing and genotyping of SNPs.

Cell lines and virus culture

SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, USA). MRC5 human fibroblasts were cultured in modified Eagle's medium (MEM) with 10% FBS and 1% nonessential amino acid (NEAA). The laboratory HCMV strain AD169 was propagated and titrated in the MRC5 cells. The virus was stored at -80°C until use. SH-SY5Y cells were infected with lentivirus containing IFITM3 wild type (Genechem, Shanghai, China) and IFITM3 NA21 (Hanbio, Shanghai, China) according to the suppliers' instructions.

Immunohistochemistry (IHC)

The brain tissue samples were fixed with 4% paraformaldehyde and embedded in paraffin. Tissue sections were subjected to immunostaining for HCMV pp65 and IFITM3. In brief, the endogenous peroxidase activity and nonspecific antibody binding sites were blocked with 3% hydrogen peroxide and 1% bovine serum albumin (BSA), respectively. Antigen retrieval was performed using citric acid buffer using a microwave oven. Subsequently, the sections were incubated at 4°C overnight with the following antibodies: anti-HCMV pp65 (ab49214, 1:200, Abcam, Cambridge, UK) or anti-IFITM3 (HPA004337, 1:200, Sigma-Aldrich, St. Louis, MO, USA), followed by the addition of a secondary HRP-conjugated antibody. The reaction was visualized by the addition of 3,3'-diaminobenzidine (DAB) as a chromogen. Once the brown color was observed under a microscope, the reaction was stopped by removing the DAB and washing with ddH_2O . Negative controls were obtained using phosphate buffer instead of the primary antibody.

In situ hybridization (ISH)

For the detection of HCMV pp65 DNA in the brain sections, a digoxin-labeled DNA probe specific for pp65 (5'-GCTCTTCTTTTTTCGATATCGACTTGTTGCTGCAGCG-3'), digoxin-labeled Alu DNA probe (positive control), and insect genomic DNA probe (negative control) were purchased from Life Technologies. Tissue sections were hybridized with the probes at 95°C for 20 min, followed by incubation for 16 h at 37°C . After incubation with

mouse anti-digoxin IgG, the reaction was visualized by the addition of HRP-conjugated goat anti-mouse IgG and DAB.

Scoring methodology of immunohistochemistry and in situ hybridization

The immunostaining results were evaluated by using a previously described scoring methodology.^{26,27} Positive cells were counted using image analysis software (Image-Pro[®] Plus 6.0; Media Cybernetics Inc.). Cells showing yellow or brown particles in the cytoplasm or nucleus were considered positive. The sections were observed under five random high-power fields, and 100 cells were counted per field. The semi-quantitative results were expressed as the percentage of positive cells combined with a subjective assessment of staining intensity. The staining intensity was scored as 0 (colorless), 1 (light yellow), 2 (yellow or brown), and 3 (dark brown); the percentages of positive cells were denoted as 0 (<5%), 1 (5–25%), 2 (26–50%), 3 (51%–75%), and 4 (>75%). The multiplication of both scores was used to evaluate the immunostaining results as follows: overall scores of 0, $1-3$, $>3-6$, and >6 were defined as negative, weakly positive, moderately positive, and strongly positive, respectively.

Double immunofluorescence

After the slides were pretreated as described in the immunohistochemical analysis section, the sections were simultaneously incubated with two primary or secondary antibodies. The primary antibodies used in the staining were as follows: mouse anti-HCMV pp65 monoclonal antibody (ab49214, 1:200, Abcam, Cambridge, UK), rabbit anti-IFITM3 polyclonal antibody (HPA004337, 1:200, Sigma-Aldrich, St. Louis, MO, USA), mouse anti-NeuN polyclonal antibody (ab104224, 1:200, Abcam, Cambridge, UK), mouse anti-GFAP polyclonal antibody (3670s, 1:200, CST, Danvers, USA), mouse anti-Iba-1 polyclonal antibody (ab15690, 1:200, Abcam, Cambridge, UK), and mouse anti-IE1 (ab65104, 1:40, Abcam, Cambridge, UK). The secondary antibodies were Alexa Fluor 488-labeled donkey anti-mouse immunoglobulin G (IgG) and Alexa Fluor 594-labeled donkey anti-rabbit IgG, 1:1000 for both (Life Technologies, USA). The nucleus was stained with DAPI, and the images were observed and recorded under a microscope (Olympus BX61, Olympus, Japan).

Sequencing and genotyping of SNPs

Blood samples were collected from 45 RE patients and 42 TLE patients. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden,

Germany) by standard methods according to the manufacturer's instructions. The region encompassing the human IFITM3 rs12252 was amplified by PCR. The forward and reverse primers were as follows: 5'-GGAACTGTTGAGAAACCGAA-3' (F) and 5'-CATACG-CACCTTCACGGAGT-3' (R). All PCR products were purified and sequenced to find T/C polymorphisms in the rs12252 region. SNPs were identified using Chromas (Technelysium Pty Ltd). The allele frequencies and genotypes of healthy Han Chinese were obtained from the 1,000 Genomes Project (<http://www.1000genomes.org>).

HCMV infection experiments in SH-SY5Y cells

In an in vitro infection experiment, SY5Y-IFITM3 cells and SY5Y- Δ 21 cells were infected with HCMV strain AD169 at a multiplicity of infection (MOI) of 1. Heat-inactivated (65°C for 30 min) HCMV was used for mock infection. After incubation at 37°C for 2 h, the virus solution was removed, and then the cell culture was continued to culture until set times at which the cells and supernatants from the mock- and HCMV-infected groups were collected for the detection of HCMV replication. Infection experiments were performed independently at least three times.

Real-time PCR

For determining levels of viral genes, total cellular RNA was extracted from cells using the TRIzol reagent (Transgen, Beijing, China) according to the manufacturer's instructions. The concentration of RNA was quantified using a NanoDrop2000c Spectrophotometer (Nano-Drop Technologies). cDNA was synthesized using 1 μ g of total RNA as the template and oligo dT as a primer, as described in the manufacturer's protocol for reverse transcription (Promega, USA). A fluorescence quantitative PCR instrument (Applied Biosystems 7500 Fast Real-Time PCR Systems, Life Technologies, Carlsbad, USA) and a GoTaq® qPCR Master Mix (Promega, USA) kit were used to detect target gene expressions. The sequences of primers are listed in Table 1. The $2^{-\Delta\Delta CT}$ method was employed to determine the relative expression of target genes normalized to β -actin. The determination for each sample was repeated three times.

Cellular immunofluorescence

At the set times, the cells were fixed with 4% formaldehyde for 15 min and washed with PBS three times. The cells were then permeabilized and blocked in PBS with 0.1% Triton-X100 followed by 2% FBS for 1 h at room

Table 1. Sequence of primers used in the PCR for HCMV.

Primer	Sequence
IE1	F: 5'-GCCTTCCCTAAGACCACCAAT-3' R: 5'-ATTTTCTGGGCATAAGCCATAATC-3'
UL44	F: 5'-GTCGGGAACAGCGGCAAGTC-3' R: 5'-CGCCACCTCCTCCAGACC-3'
UL94	F: 5'-TGACGCAGTCGCTCATCCACAAC-3' R: 5'-CCAGGTCCACACATCGGTCTTG-3'
β -actin	F: 5'-TGGCACCCAGCACAAATGAA-3' R: 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'

temperature. After blocking, the samples were simultaneously incubated with rabbit anti-IFITM3 polyclonal antibody (HPA004337, 1:200, Sigma-Aldrich, St. Louis, MO, USA) and mouse anti-IE1 (ab65104, 1:40, Abcam, Cambridge, UK). The secondary antibodies were Alexa Fluor 488-labeled donkey anti-mouse immunoglobulin G (IgG) and Alexa Fluor 594-labeled donkey anti-rabbit IgG, 1:1000 for both (Life Technologies, USA). Moreover, incubation with Alexa Fluor 488-labeled donkey anti-mouse immunoglobulin G (IgG) and Alexa Fluor 594-labeled donkey anti-rabbit IgG was simultaneously carried out for 30 min at room temperature. Finally, DAPI was used for nuclei counterstaining, and images were obtained using fluorescent microscopy.

Statistical analysis

Statistical analysis was performed using SPSS 17.0 (SPSS Inc.). The counting data were tested by chi-square test or Fisher's exact probability method. Allelic and genotypes of individuals were tested for Hardy-Weinberg equilibrium (HWE). Difference between groups was evaluated using chi-square test, and odds ratio (OR) together with 95% confidence intervals (CIs) were estimated. The mean \pm standard deviation was used to represent the measurement data under the assumption of a normal distribution. Comparisons between multiple groups were performed using one-way analysis of variance (ANOVA) or the unpaired Student's *t*-test. Differences were considered significant at $P < 0.05$.

Results

The expression of HCMV components is elevated in RE brain tissue

To explore the role of HCMV infection in the pathogenesis of RE, the expression of HCMV components in RE brain tissue was detected by IHC and ISH. As shown in Figure 1, positive immunostaining for pp65 protein or DNA was predominantly observed in the cytoplasm and/

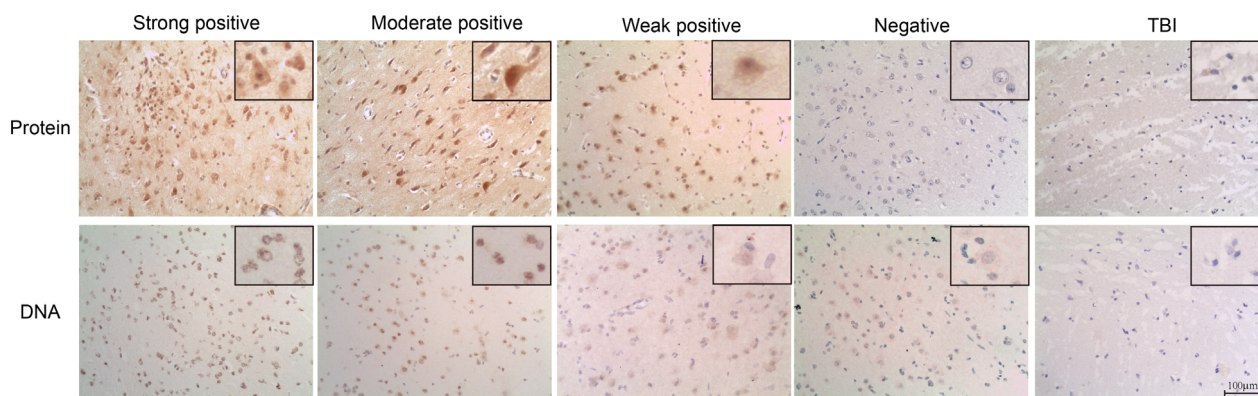


Figure 1. Expression of HCMV in RE brain tissue. HCMV pp65 protein and DNA were detected by IHC and ISH, respectively. Representative images are shown at low or high magnification. Positive staining of pp65 was predominantly observed in the cytoplasm and nuclei of cells.

or nucleus of neuron-like cells. Of the 45 RE patients, 35 cases (77.78%) showed positive immunostaining for HCMV pp65 protein. Among these patients, 15 cases (33.33%) were strongly positive, 13 cases (28.89%) were moderately positive, and 7 cases (15.56%) were weakly positive (Table 2). Accordingly, HCMV pp65 DNA was detected in 71.11% (32/45) of RE cases, and 14 (31.11%), 15 (33.33%), and 3 (6.67%) were strongly, moderately, and weakly positive, respectively (Table 2). In the control group (non-RE), only two cases (2/20, 10%) were weakly positive for the pp65 antigen, and all were negative for pp65 DNA. There were significant differences in the detection of pp65 protein and DNA between the two groups ($P < 0.05$ for both). Notably, all 32 HCMV pp65 DNA positive RE samples were positive for HCMV pp65 protein. These results suggested that HCMV components are highly expressed in RE brain tissue and may be involved in the pathogenesis of RE.

Table 2. Expression of HCMV in the brain tissue of RE patient.

	IHC		ISH	
	RE (n = 45)	Non-RE (n = 20)	RE (n = 45)	Non-RE (n = 20)
Positive	35 (77.78%)	2 (10%)	32 (71.11%)	0
Strong	15 (33.33%)	0	14 (31.11%)	0
Moderate	13 (28.89%)	0	15 (33.33%)	0
Weak	7 (15.56%)	2 (10%)	3 (6.67%)	0
Negative	10 (22.2%)	18 (90%)	13 (28.89%)	20 (100%)

The expression of IFITM3 is elevated in RE brain tissue

Virus infection has been determined to activate innate immune-related gene expression in the host. IFITMs, especially IFITM3, can be activated upon infection by a range of pathogenic viruses. To investigate the role of IFITM3 in the pathogenesis of RE, its expression was detected in the brain tissue of RE patients by IHC. As shown in Figure 2A, the positive staining of IFITM3 was distributed mainly in the peri-nucleus of the neuron-like cells. A total of 63.33% of RE brain sections showed positive immunostaining for IFITM3. The strong, moderate, and weak positive staining rates were 16.67%, 16.67%, and 30%, respectively (Fig. 2B). In contrast, the expression of IFITM3 was barely detected in the control brain tissue. Double immunofluorescence staining in RE brain sections further showed IFITM3 to be colocalized with NeuN, a molecular marker for neurons, but not with GFAP or Iba-1, the molecular markers for astrocytes and microglia, respectively (Fig. 3A). The colocalization indicated that IFITM3 was expressed primarily in neurons in the RE brain. Moreover, the colocalization of HCMV and IFITM3 was also detected in the RE brain (Fig. 3B). These results suggested that HCMV infection might induce the expression of IFITM3 in neurons and that IFITM3 is involved in the immune response against HCMV infection in RE brain tissue.

Genotype distribution and allele frequency of IFITM3 rs12252 in RE patients

To determine whether IFITM3 rs12252 is associated with RE, we sequenced 300 base pairs of the IFITM3 locus encompassing SNP rs12252 in 45 RE patients. Among these patients, 44.44% (20/45) of RE patients carried the CC genotype, a higher frequency than in Han Chinese in

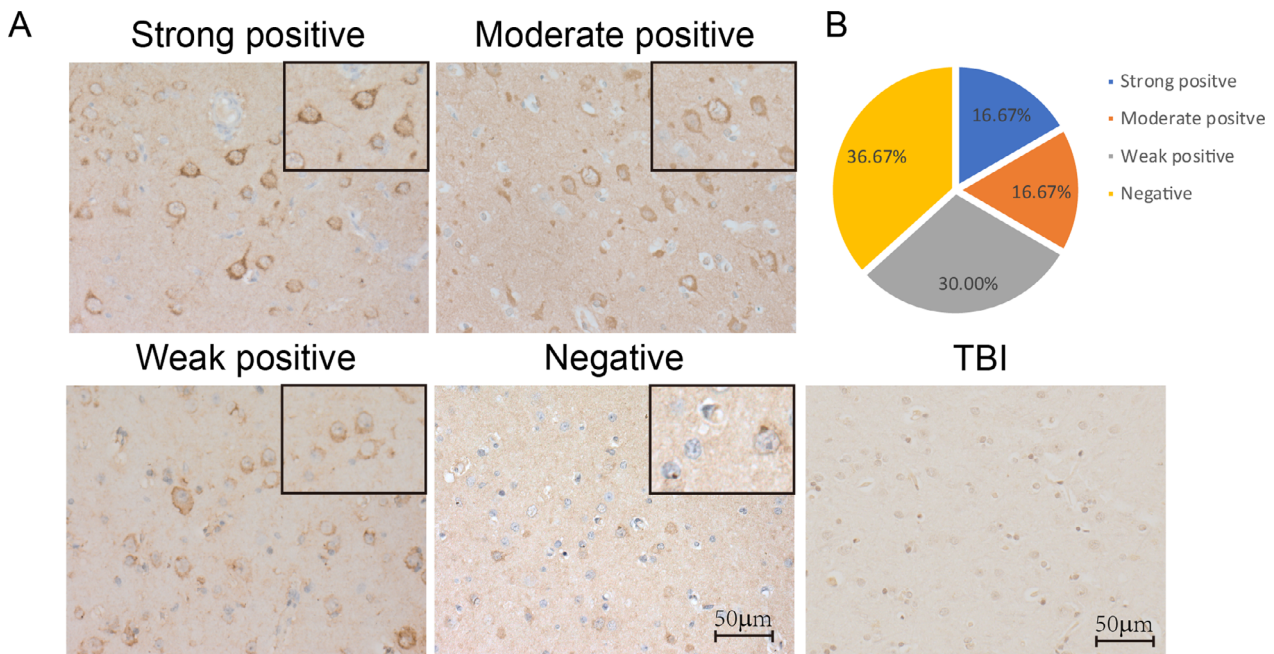


Figure 2. Expression of IFITM3 in RE brain tissue. (A) IFITM3 protein was detected by IHC in RE brain tissue. Representative images are shown at low or high magnification. Positive staining of IFITM3 was predominantly observed in the cytoplasm of cells. (B) Statistical analysis of IFITM3 expression in RE brain tissue.

the 1,000 Genomes Project sequence database (26.92% CC genotype; $P < 0.05$, chi-square test) (Table 3). We measured a 40% (18/45) CT genotype and a 15.56% (7/45) TT genotype in the other RE cases. The C allele frequency in the RE patients was 64.44%, which was significantly higher than in the general population of Han Chinese. The IFITM3 SNPs were in HWE ($P > 0.05$). Odds ratios and 95% CIs were used to assess the association strength of the IFITM3 rs12252 polymorphism and RE. The results indicated an increased risk of RE in subjects carrying the IFITM3 rs12252 CC genotype (OR = 2.17, 95% CI = 1.12–4.21). These results suggested that the IFITM3 rs12252 may be closely associated with RE disease occurrence.

IFITM3 polymorphism rs12252-C associated with HCMV infection in RE patients

Next, we further analyzed the correlation between IFITM3 rs12252 CC genotype and the expression of HCMV antigen in RE brain tissue. According to the IHC results of HCMV antigen staining, the 45 RE patients were divided into positive and negative groups. It was found that 22 patients (62.86%) in the HCMV-positive group carried the CC genotype and that 2 patients (20%) in the HCMV negative group carried the CC genotype. There was a significantly higher frequency of the CC genotype in the

HCMV-positive patients than in the HCMV-negative patients (62.86% vs. 20%, $P < 0.05$) (Fig. 4A). The frequency of the C allele in the HCMV-positive patients was significantly higher than in the HCMV-negative patients (78.57% vs. 40%, $P = 0.001$, Fig. 4A). These results suggested that the IFITM3 rs12252 C polymorphism is correlated with the expression level of HCMV in the brain tissue of RE patients.

IFITM3 polymorphism rs12252-C is associated with clinical progression of RE patients

To uncover the role of the IFITM3 rs12252 CC genotype in the development of RE, we analyzed the association of IFITM3 rs12252 polymorphisms and primary clinical characteristics in RE patients. Since progressive brain atrophy is a major characteristic of RE, we divided the degree of cerebral atrophy in RE patients into different grades (scored 0–3) according to MRI images. In RE patients carrying the CC and CT/TT genotypes, the average MRI scores were 2.41 and 1.78, respectively. The average MRI scores in the RE patients carrying allele C and allele T were 3.22 and 2.19, respectively (Fig. 4B). The degree of brain atrophy in patients carrying the CC genotype was significantly higher than in patients carrying the CT or TT genotypes ($P < 0.01$), and the degree of brain

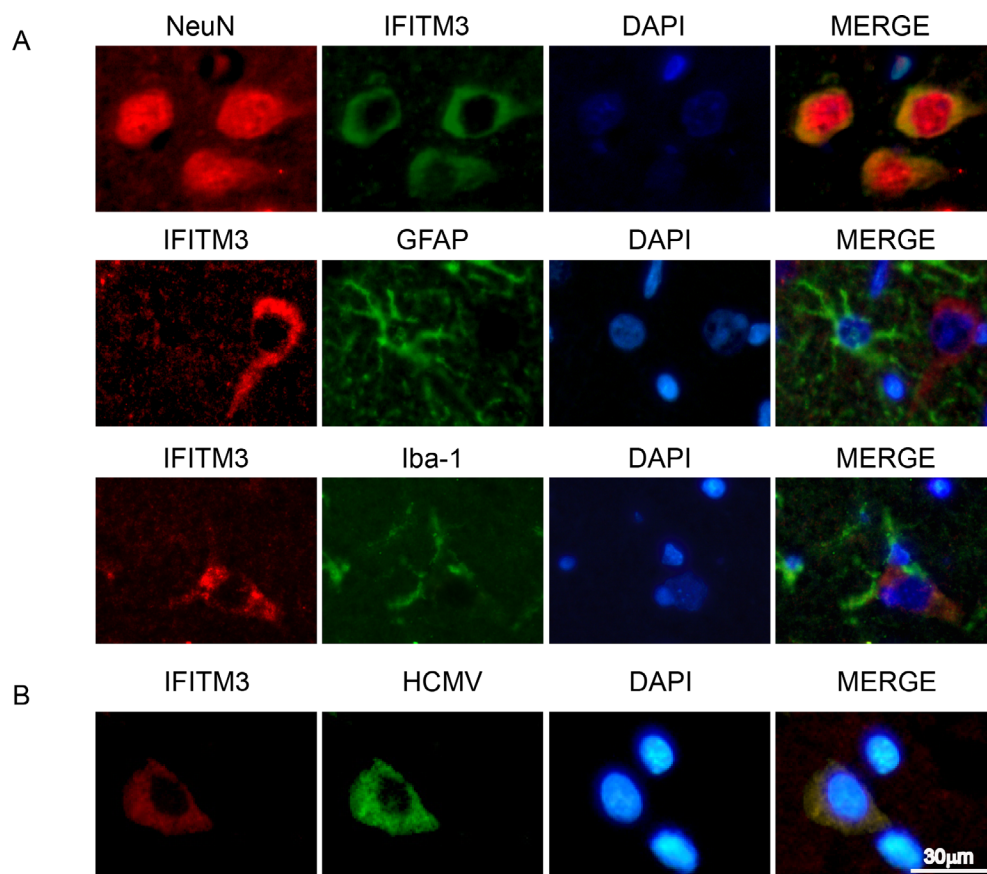


Figure 3. Immunofluorescence staining demonstrating the cellular distribution of IFITM3 in brain sections. (A) Costaining of IFITM3 with NeuN (a neural marker), GFAP (an astrocyte marker), and Iba-1 (a microglial marker). (B) Costaining of IFITM3 with HCMV pp65. Merged images show colocalization of the green and red labels. Nuclei are labeled with DAPI.

Table 3. Genotype distribution and allele frequency of IFITM3 rs12252 in RE patients.

Type		RE		TLE		Han Chinese
CC	20	44.44%		11	26.19%	56
CT	18	40.00%		21	50.00%	105
TT	7	15.56%		10	23.81%	47
Allele C	58	64.44%		43	51.19%	217
Allele T	32	35.56%		41	48.81%	199
Statistical analysis						
Genotype						
	<i>P</i> -value	0.02*		0.92		
	Odds ratio (95%CI)	2.17 (1.12–4.21)		0.96 (0.45–2.04)		
	HWE <i>P</i> -value	0.39*		0.99		
Allele						
	<i>P</i> -value	0.037*		0.87		
	Odds ratio (95%CI)	1.65 (1.03–2.64)		0.96 (0.60–1.54)		

**P* < 0.05.

atrophy in patients carrying allele C was significantly higher than that in patients carrying allele T (Fig. 4B, *P* < 0.05). These results suggested that brain atrophy was

more severe in RE patients carrying the IFITM3 rs12252 CC genotype and allele C than in others. In other words,

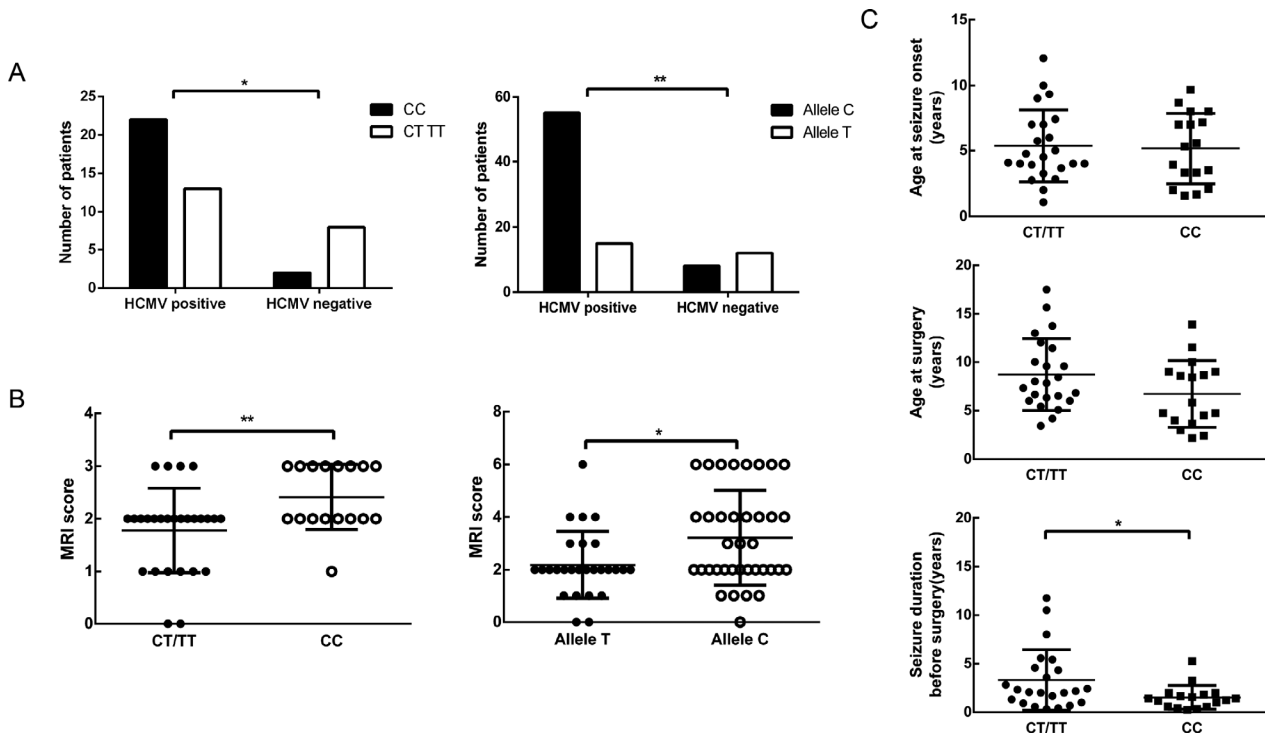


Figure 4. Associations of IFITM3 rs12252 polymorphism with pathological and clinical characteristics in RE patients. (A) Association of IFITM3 rs12252 polymorphism with HCMV expression in brain tissue. Forty-five RE patients were divided into HCMV-positive and HCMV-negative groups based on the IHC scoring of HCMV pp65 staining (* $P < 0.05$, ** $P < 0.01$). (B) Association of the genotype frequency of IFITM3 rs12252 with brain atrophy in RE patients. MRI scoring: 0 means no atrophy and no ventricle or sulcus enlargement, 1 means weak atrophy with the sulci deepening without ventricle enlargement, 2 means moderate atrophy and sulci deepening with moderate ventricular dilatation of less than half of the contralateral ventricular dilatation, and 3 means severe atrophy and sulci deepening with severe ventricular dilatation of more than half of the contralateral ventricular dilatation. (C) Association of the genotype frequency of IFITM3 rs12252 with the age at seizure onset, age at surgery and seizure duration before surgery in RE patients.

IFITM3 rs12252 CC was associated with the severity of RE.

As the main clinical manifestation of RE patients is refractory seizures, we analyzed the association of the IFITM3 rs12252 polymorphism and clinical seizure characteristics. As shown in Figure 4, the average age at seizure onset of patients carrying the CC and CT/TT genotypes was 5.16 ± 0.65 years and 5.37 ± 0.57 years, respectively (Fig. 4C, $P = 0.82$), and the average age at surgery of patients carrying the CC and CT/TT genotypes was 6.72 ± 0.83 years and 8.71 ± 0.77 years, respectively (Fig. 4C, $P = 0.09$). There was no difference in the age at seizure onset or age at surgery between the two groups. The seizure duration before surgery in patients carrying CC and CT/TT genotypes was 1.53 ± 0.30 years and 3.33 ± 0.65 years, respectively, and there was a significant difference between the two groups (Fig. 4C, $P < 0.05$). These results suggested that RE patients carrying the CC genotype undergo faster disease progression than RE

patients carrying the CT/TT genotypes, as correlated with the RE disease severity.

IFITM3 polymorphism rs12252-C is associated with HCMV infection in SH-SY5Y cells

To further verify the effect of the IFITM3 rs12252 polymorphism on HCMV infection, the SH-SY5Y cell line was transfected with wild-type IFITM3 (SY5Y-IFITM3) and the mutant with 21-amino-acid deletion (SY5Y- Δ 21) by lentiviral vectors and then infected by HCMV AD169. The expression of HCMV viral lytic replication genes, as determined by real-time PCR, was used to evaluate viral replication. Compared with the control group, the mRNA levels of HCMV IE1 (early gene), UL44 (medium gene), and UL94 (late gene) decreased in SY5Y-IFITM3 cells and increased in SY5Y- Δ 21 cells at 1, 2, and 3 days postinfection (dpi). These values were

significantly different from those of the corresponding controls (Fig. 5A and B, $P < 0.05$ or $P < 0.01$). Compared with SY5Y-IFITM3 cells, the mRNA levels of above-mentioned genes increased significantly in SY5Y-NΔ21 cells (Fig. 5C).

The expression of IFITM3 and HCMV IE1 was also examined using immunofluorescence staining. As show in Figure 6A, both full length IFITM3 and NΔ21-IFITM3 localized in the membrane and endosomes of SH-SY5Y cells. Specific fluorescence for the IE1 protein was observed in the SY5Y-IFITM3 and SY5Y-NΔ21 cells at 12 h after HCMV infection (Fig. 6B). In line with the changes in the IE1 mRNA levels, the percentage of HCMV IE1-positive cells decreased from 21.55% to 7.43% at 1 dpi and from 23.62% to 12.99% at 2 dpi in the SY5Y-IFITM3 cells (Fig. 7B). In contrast, the percentage of HCMV IE1-positive cells increased from 21.28% at 1 dpi to 28.07% and from 21.72% to 44.38% at 2 dpi in the SY5Y-NΔ21 cells, which was accompanied by increased immunofluorescence intensity (Fig. 7C). Compared with SY5Y-IFITM3 cells, the number of HCMV IE1

positive cells significantly increased in SY5Y-NΔ21 cells (Fig. 7D), indicating that IFITM3 could restrict HCMV infection and the rs12252 SNP would lose the antiviral ability at least in part.

Discussion

When RE was first reported, viral infection was speculated as an important factor in the pathogenesis. Although several viral antigens were detected in RE brain tissue, it is hard to find a specific link between the viruses detected in brain tissue and disease progression. In this study, by detecting the expression of HCMV antigens and IFITM3 and analyzing the frequency of IFITM3 rs12252 polymorphism in 45 RE patients, we report for the first time a combined effect of HCMV infection and genetic susceptibility in RE disease progression.

IFITM3 can be upregulated by IFN to be involved in the host's immune response against multiple viruses. It has been found to inhibit the replication of several RNA viruses, including the influenza virus, West Nile virus,

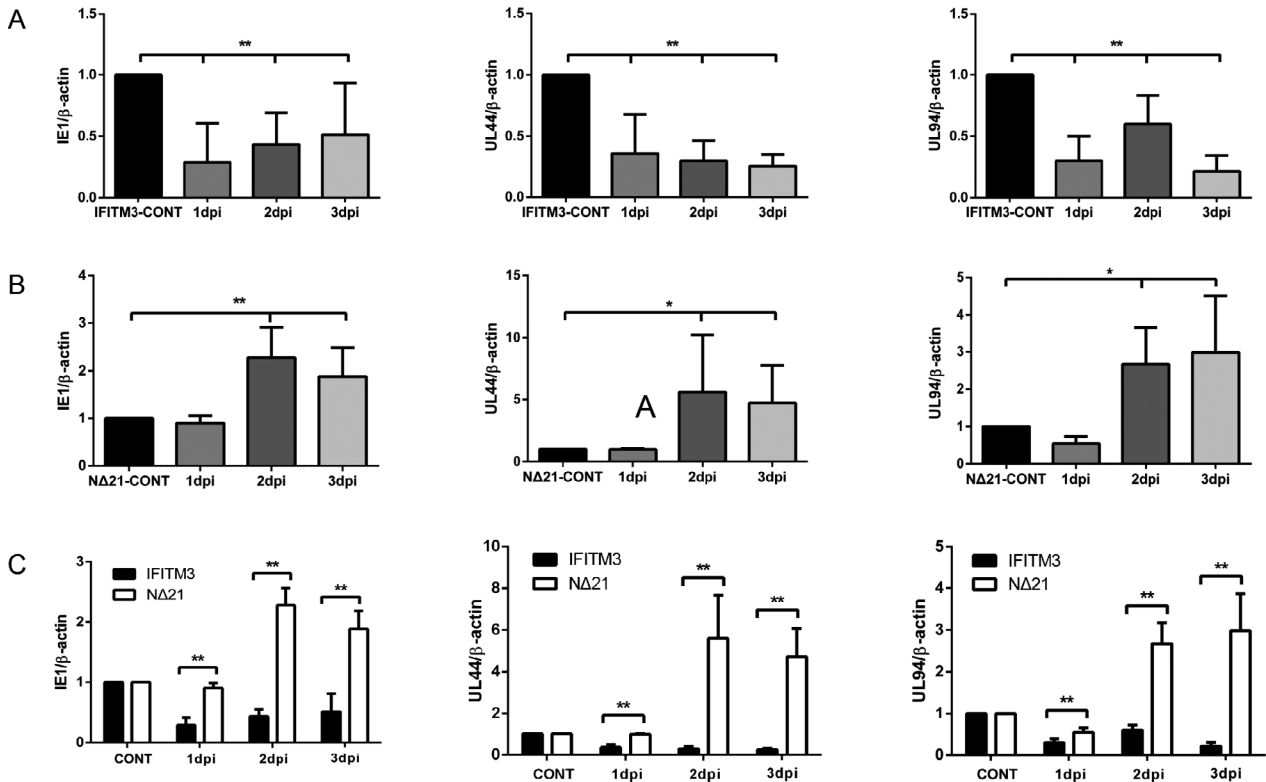


Figure 5. Effect of IFITM3 rs12252 polymorphism on HCMV gene expression in SH-SY5Y cells. Full-length IFITM3 and N21T-IFITM3 were overexpressed in SH-SY5Y cell. HCMV AD169-infected SY5Y-IFITM3 and SY5Y-N21T at MOI = 1. qRT-PCR was used to analyze the expression of HCMV lytic gene expression (immediate early, early, and late gene) in SY5Y-IFITM3 and SY5Y-N21T after HCMV infection (* $P < 0.05$, ** $P < 0.01$).

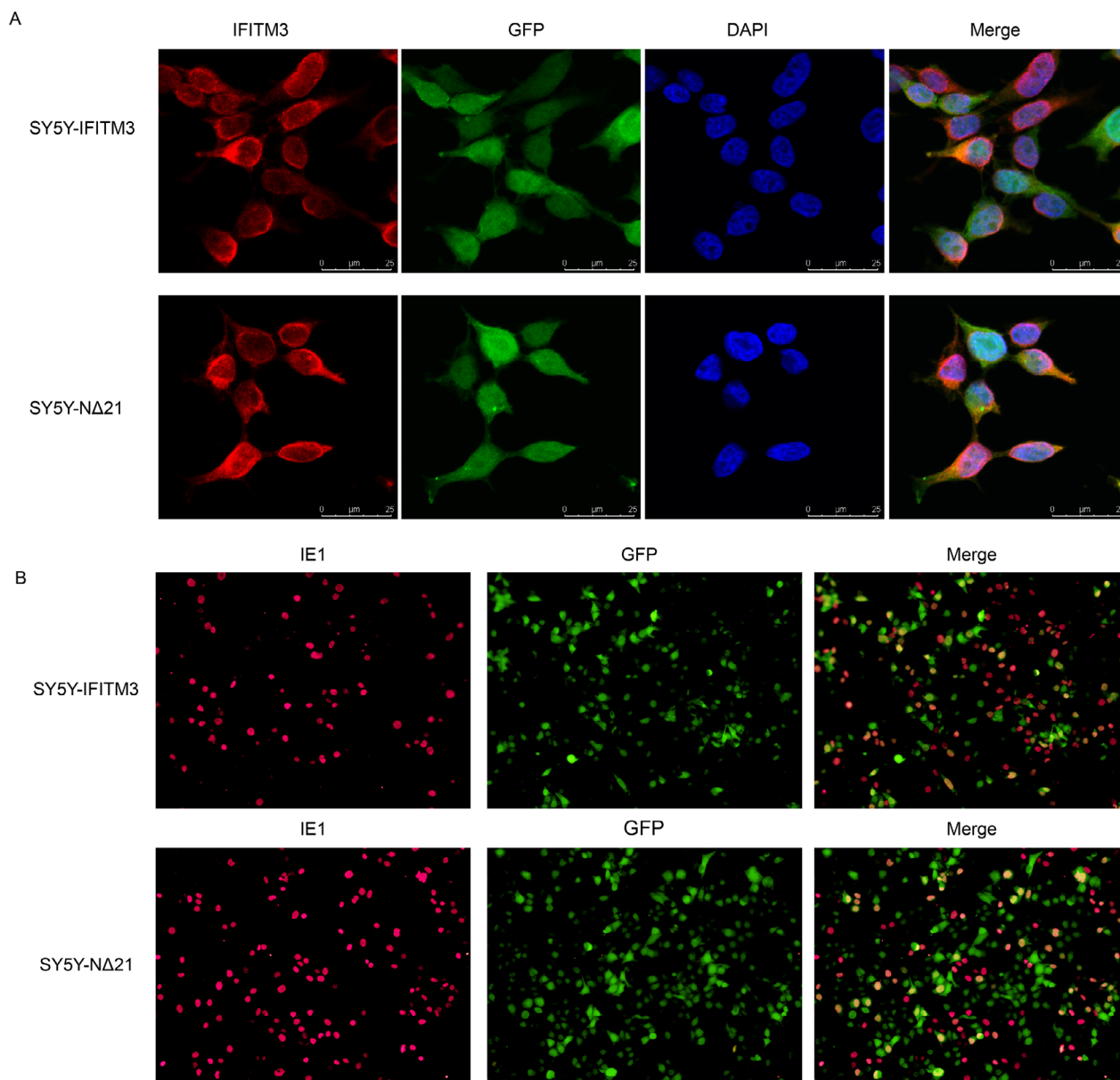


Figure 6. Expression of IFITM3 and HCMV proteins in SH-SY5Y Cells. (A) Subcellular localization of IFITM3 in SH-SY5Y cells transfected with lentivirus containing IFITM3 and NΔ21 IFITM3. The vectors included green fluorescence protein (GFP) reporter gene. In GFP positive cells, full length IFITM3 and NΔ21 IFITM3 could be stably expressed. (B) Cell immunofluorescence analysis show HCMV protein expressed in SY5Y-IFITM3 and SY5Y-NΔ21 cells at 12 h after virus infection.

Ebola virus, and Zika virus.^{19,28–30} However, for the MERS virus, which is also a respiratory RNA virus, IFITM3 can promote the virus replication.³¹ The effect of IFITM3 on DNA virus infection has been less thoroughly studied. A few studies reported the effect of IFITM3 on CMV replication. Warren et al. concluded that IFITM3 did not affect HCMV replication *in vitro*.³² However, the others found that IFITM3 inhibited HCMV replication by inhibiting virus assembly in MRC5 cells²⁴ and prevented

cytokine-driven CMV pathogenesis.²⁵ In the present study, it was found that in RE brain tissue, the expression of IFITM3 was upregulated and it colocalized with HCMV in virus-infected neurons, indicating a possible interaction between IFITM3 and HCMV in the brain of RE patients.

IFITM3 is known to lose its antiviral function due to its gene mutation. Several SNPs have been identified in IFITM3.³³ Among them, IFITM3 rs12252 polymorphism

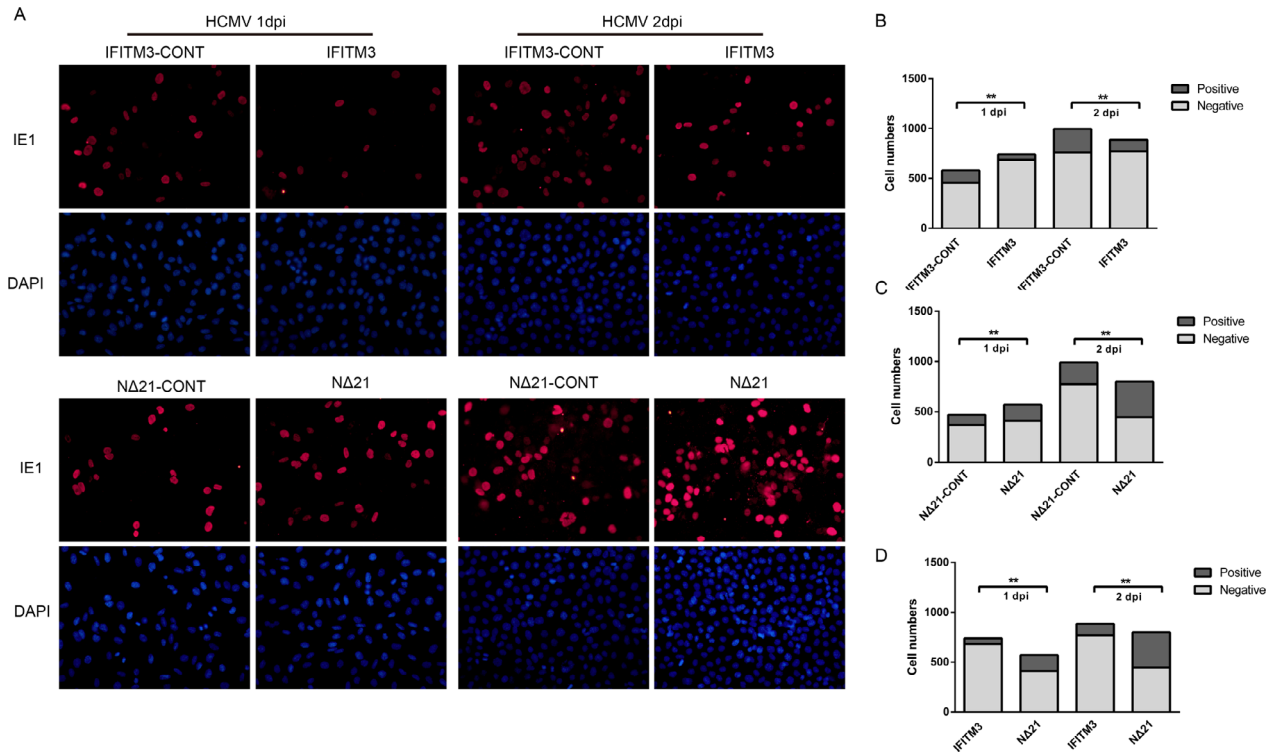


Figure 7. Effect of IFITM3 rs12252 polymorphism on HCMV protein expression in SH-SY5Y cells. (A) Cell immunofluorescence analysis was used to detect the HCMV infection rate in SY5Y-IFITM3 and SY5Y-N21T at 1 dpi and 2 dpi. (B–D) The number of HCMV IE1-positive cells was counted in five random fields under the microscope, and the percentage of HCMV IE1-positive cells was calculated as the number of positive cells divided by the total number of counted cells (* $P < 0.05$, ** $P < 0.01$).

was reported to be closely associated with severity and outcome of some infection diseases. Therefore, we identified the frequency of the IFITM3 rs12252 polymorphism in RE patients. The frequency of the CC genotype and allele C was more common in the RE group than in the Han Chinese. Interestingly, there is a similar IFITM3 rs12252 genotype distribution to Han Chinese in TLE patients, the most common type of partial onset epilepsy in the clinic, indicating a link between the IFITM3 rs12252 polymorphism and RE. Furthermore, we found that IFITM3 rs12252-C is associated with high detection of HCMV in RE brain tissue and rapid progression of the disease indicated by short time period from first epilepsy onset to surgical control of clinical epilepsy. In combination with above data, we suggested that the IFITM3 polymorphism rs12252-C may associate with susceptibility to HCMV infection in RE brain tissue and individuals carrying the C allele were found to have a high risk of HCMV infection. The link between the rs12252 SNP and HCMV infection may be an important cause of RE. Intrinsic host susceptibility to viral infections plays a major role in determining infection severity in different individuals. Genetic diversity within IFITM3 determines the susceptibility to HCMV infection in RE patients.

To further determine effect of IFITM3 on HCMV infection, we used SH-SY5Y cells to establish cell line overexpressing full-length IFITM3 or Δ21 IFITM3 in this study. We found that IFITM3 significantly inhibited HCMV replication revealed by decreased IE1, UL44, and UL94 gene levels while Δ21 IFITM3 promoted the replication of those genes compared with the control results, which were further supported by IFA staining. Those results suggested that IFITM3 effectively restricted HCMV infection and Δ21 IFITM3 loss the anti-HCMV activity. Previously, it was found that Δ21 IFITM3 isoform inhibited replication of influenza A virus via blocking the entry.³⁴ Xie et al. reported that IFITM3 restricted HCMV replication by inhibiting virus assembly.²⁴ Moreover, viral disease is accompanied by elevated production of cytokines in IFITM3 knock out mice and IFITM3 represents a checkpoint regulator of antiviral immunity that controls cytokine production to restrict viral pathogenesis.²⁵ It is speculated that inflammatory gene expression may be a secondary effect of a primary immune response.

There are some limitations in this study. The histopathological features were often observed in unilateral cerebral hemisphere of RE patients while the IFITM3 rs12252 polymorphism and HCMV infection should

occur in the whole body. The results in present study cannot explain mechanism underlying unilateral affection of cerebral hemisphere of RE patients. HCMV was detected in the affected unilateral RE brain tissue. We do not know whether this property of HCMV infection may contribute to unilateral affection in RE patients because we are unable to examine the presence of HCMV in the other hemisphere of RE patients. As mentioned above, these results can give an explanation why RE patients are more susceptible to virus infection. In addition to virus infection, other factors must associate with this special RE pathogenesis. Establishing an animal model for RE is urgently required to clarify this issue.

Conclusion

This study demonstrated association between IFITM3 rs12252 polymorphisms and disease progression in RE patients. The results provide new insight into understanding the pathogenesis of RE.

Conflict of Interest

None of the authors disclose any conflict of interest concerning this manuscript.

Authors' Contributions

JA, GL, and YW designed the study. YG and GL collected clinical samples and data. YW performed analyses on IHC, double immunofluorescence, and genotyping. YW, YG, and GL performed analyses on the clinical disease progression. YW and QL performed analyses on qPCR and cellular immunofluorescence. JA and YW interpreted the results. YW and YG performed statistical analysis. JA and YW drafted the manuscript. DF participated in the coordination of the study. All authors have read and approved the final manuscript.

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