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Research paper

The NS3 and NS4A genes as the targets of RNA interference inhibit replication of Japanese encephalitis virus in vitro and in vivo

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ABSTRACT

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus that can cause acute encephalitis with a high fatality rate. RNA interference (RNAi) is a powerful tool to silence gene expression and a potential therapy for virus infection. In this study, the antiviral ability of eight shRNA expression plasmids targeting different sites of the NS3 and NS4A genes of JEV was determined in BHK21 cells and mice. The pGP-NS3-3 and pGP-NS4A-4 suppressed 93.9% and 82.0% of JEV mRNA in cells, respectively. The virus titer in cells was reduced approximately 950-fold by pretreating with pGP-NS3-4, and 640-fold by pretreating with pGP-NS4A-4. The results of western blot and immunofluorescence analysis showed JEV E protein and viral load in cells were remarkably inhibited by shRNA expression plasmids. The viral load in brains of mice pretreated with pGP-NS3-4 or pGP-NS4A-4 were reduced approximately 2400-fold and 800-fold, respectively, and the survival rate of mice challenged with JEV were 70% and 50%, respectively. However, the antiviral ability of shRNA expression plasmids was decreased over time. This study indicates that RNAi targeting of the NS3 and NS4A genes of JEV can sufficiently inhibit the replication of JEV in vitro and in vivo, and NS3 and NS4A genes might be potential targets of molecular therapy for JEV infection.

1. Introduction

Japanese encephalitis virus (JEV), belonging to the *Flavivirus* genus of the *Flaviviridae* family, can cause serious lesions of the central nervous system of humans, stillbirths in sows, and orchitis in breeding boars, and JEV can spread from swine to humans through mosquito bites (Wang & Liang, 2015). JEV is the main cause of viral encephalitis in Asia. According to the latest report, 67,900 human JEV infections occur annually in endemic countries, with approximately 51,000 (75%) occurring in children aged 0 to 14 years (Campbell et al., 2011). At present, there are no specific drugs or therapies for the treatment of JEV infection. JEV has a single-stranded, plus-sense RNA genome contains a 5′ non-coding region (UCR), followed by a 10,296-nucleotide coding region and a 3′ UCR. The only open reading frame (ORF) codes a large polyprotein which is cleaved into three structural and seven non-structural proteins by viral and cellular proteases (Solomon, 2004).

In the process of RNA interference (RNAi), long dsRNA is processed by the enzyme dicer into small interfering RNAs (siRNAs, 21 to 25 nt), which induce a sequence-specific degradation of the target mRNA (Wilson & Doudna, 2013). Meanwhile, the interferon (IFN) response in mammalian cells induced by long dsRNA could result in cell death. However, because short synthetic dsRNA resembles the dicer-processed product, it could mediate specific gene silencing in mammalian cells without the IFN response (Elbashir et al., 2001). Currently, the siRNAs are becoming powerful investigational tools and potential therapeutics that allow selectively silenced gene expression in living organisms via the RNAi pathway (Dornseifer et al., 2015).

Currently, there are no specific antiviral drugs for the treatment of JEV-associated disease. The molecular therapy of JEV infection by RNAi may be a novel antiviral strategy but further development is needed. Recent studies have demonstrated that RNAi can induce the effective inhibition of flaviviruses in vitro and in vivo, such as the tick-borne encephalitis virus, dengue virus, classical swine fever virus, hepatitis C

Abbreviations: JEV, Japanese encephalitis virus; DENV, dengue virus; HCV, hepatitis C virus; BVDV, bovine viral diarrhea virus; NS3, non-structural 3 protein; C, capsid protein; M, membrane protein; E, envelope protein; min, minute; h, hour; hpt, hours post transfection; hpi, hours post infection; shRNA, short hairpin RNA; RT-PCR, reverse transcription-polymerase chain reaction; PBS, Phosphate buffer solution; DMEM, Dulbecco's modification of Eagle's medium; MOI, multiplicity of infection; PFU, plaque forming unit; LD_{50}, 50% lethal dose.

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virus and West Nile virus (Achazi et al., 2012; Li et al., 2010; Yun et al., 2009; Suhy et al., 2012). Studies have demonstrated that treatment with the shRNA plasmid targeting the JEV E gene could significantly inhibit viral replication in BHK-21, SK-N-SH cells and mice (Shen et al., 2014). Meanwhile, the RNAi of JEV NS5 had significant antiviral activity, JEV titers were suppressed by 99% in human embryonic kidney cells pretreated with shRNA plasmid targeting NS5 (Anantpadma & Vrati, 2012). The NS3 and NS4A proteins are the non-structural proteins of JEV. The NS3 protein has multiple enzymatic activities and plays an important role in the process of virus replication. The NS4A protein is a small membrane-bound protein involved in the viral replication complex, and the N-terminal region ofNS4A is a cofactor of NS3 that regulates the ATPase activity of NS3 (Shiryaev et al., 2009; Yun et al., 2009).

The synthetic shRNA gene cassette cloned into a plasmid to construct a shRNA expression vector, which encodes the shRNA that is processed by dicer in the cell into siRNA, can continuously suppress target gene expression (Hajeri & Singh, 2009). In this study, eight different sites of the NS3 and NS4A genes were selected as the silencing targets and the corresponding shRNA expressing plasmids were constructed. The results proved that the replication of JEV can be suppressed effectively in BHK21 cells and mice by shRNA expression plasmids. Furthermore, the mice inoculated with plasmids could be partly protected to avoid the lethal induced by JEV. This study indicates that RNAi is a practicable method to suppress JEV infection and NS3 and NS4A genes could be the potential targets.

Ethics statement

All procedures performed in this study involving animals had been approved by the Institutional Animal Care and Use Committee of Sichuan Agriculture University (Approval Number BK2015-023), Sichuan, China and followed National Institutes of Health guidelines.

2. Materials and methods

2.1. Cell lines, virus and antibodies

Baby hamster kidney (BHK21) cell were purchased ATCC company and cultured in dulbecco's modified eagle medium (DMEM) added 10% (*V*/V) calf serum, 100 U penicillin/ml and 100 mg streptomycin/ml at 37 °C with 5% CO_2. Kunming mice were purchased from Chengdu Institute of Biological Products (Chengdu, China), were maintained in animal holding laboratory under controlled condition with temperature of 25 ± 10 °C, humidity of 40 ± 10% and had free access to standard mouse diet and water. JEV strain SCYA201201 and anti-Japanese encephalitis virus rabbit polyclonal antibody was maintained in Research Center of Swine Disease, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu city, China. Anti-Japanese encephalitis virus E glycoprotein mouse monoclonal antibody was purchased from Abcam company, Burlingame, CA, USA. Lipofectamine™ 2000 Transfection Reagent was purchased from Invitrogen Corporation, Carlsbad, CA, USA.

2.2. shRNAs target sequence selection and plasmids construction

With reference to the gene sequence of the SCYA201201 strain (GenBank NO. KM658163), eight candidate target sequences in the NS3 and NS4A genes were selected (Table 1). The shRNA expression plasmids pGPU6/GFP/Neo-shRNA were constructed, and nucleotides from the wild-type shRNA, pGPU6/GFP/Neo-shNC, was used as a negative control.

2.3. Cell transfection and virus infection

The 2 μg shRNA expressing plasmids mixed with Lipofectamine 2000 were transfected into BHK21 cells in six-well plates (2×10^5 cells per well). After 4 h further incubation at 37 °C the liquid mixtures were discarded, and cells were refreshed with 2 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) calf serum per well and further cultivated at 37 °C. To ascertain the transfection efficiency, the mRNA of GFP was detected by quantitative RT-PCR, and each test was repeated three times. At 24 h post transfection (hpt), cells were infected with the SCYA201201 strain (MOI = 0.5) following cultivation in fresh medium (containing 2% fetal bovine serum) at 37 °C.

Table 1
RNAi targeting sequences and sites.

Gene	Site	Target sequences
pGP-NS3-1	4656–4676	GGACACCACCACAGGAGTTTA
pGP-NS3-2	4827–4847	GGAAGACCGCATAAGCTATGG
pGP-NS3-3	4916–4936	GGAAACCTGCAGTGAATATCC
pGP-NS3-4	5361–5381	GCATCAGGGAAATGAAATAGT
pGP-NS4A-1	6497–6517	GCATGCCTGAGCATTTCATGG
pGP-NS4A-2	6507–6527	GCATTTCATGGGAAAGACACG
pGP-NS4A-3	6587–6607	GCATGGCTCTTGAAGAACTGC
pGP-NS4A-4	6659–6679	GAGGATTCTTCCTGCTCATGA

2.4. Quantitative RT-PCR, western blot and plaque reduction test

The cell cultures were harvested at 72 h post infection (hpi) with JEV, and total RNA were extracted for quantitative RT-PCR base on a pair of primers (Table 2) targeting the E gene of JEV, meanwhile GAPDH was used as the housekeeping gene. In addition, the virus cultures were centrifuged at 3000*g* for 3 min at 4 °C, the supernatants were discarded and the cells were resuspended in 200 μl RIPA lysis buffer containing PMSF (1 mM) and laid on ice for 15 min following collection of the supernatants after centrifuging. The E protein of JEV was detected by western blot using anti-JEV E protein mouse monoclonal antibody. In addition, the virus cultures were harvested at 72 hpi to measure the virus titer by plaque reduction test, and each test was repeated three times. Then, the BHK21 cells in six-well plate were infected with JEV at 24 hpt, and the JEV were detected by indirect immunofluorescence analysis (IFA) using a rabbit polyclonal anti-JEV antibody 48 h after infection. In the consideration of the security of RNAi and reasonability of tests, the E gene of virus cultures was amplified and sequenced to determine whether there were mutations induced by RNAi.

2.5. Inoculation and challenge of suckling mice

Suckling mice (7-day-old) were divided into 11 groups (n = 6 each) and each mouse received intracerebrally inoculation of 25 μl mixed liquid containing 2 μg shRNA expression plasmids and 3 μl Lipofectamine 2000, with an equal volume of DMEM as a mock control. Then suckling mice were challenged intracerebrally with the SCYA201201 strain ($10LD_{50}$) 24 h after inoculation of plasmids. The suckling mice were sacrificed five days after challenge and the brains were excised and homogenized in DMEM to produce 10% (w/v) suspensions which were tested by plaque reduction experiment to measure the virus titer.

2.6. Protective rates of plasmids against JEV

The two most highly efficient plasmids that target NS3 and NS4A, respectively, were picked out for mice infection experiments. Three-

Table 2
Primers for quantitative RT-PCR.

Primer	Gene	Sequence (5′–3′)	Amplification size (bp)
P1	E of JEV	CATTGGAGCCACTTGGGTG	201
P2		TTGTGGGCTTCTCCTGTCG	
P3	GAPDH of BHK21	TGCCCAGAACATCATCCCT	191
P4		ATGCCTGCTTCACCACCTT	