



Genomic changes in an attenuated genotype I Japanese encephalitis virus and comparison with virulent parental strain

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Received: 26 December 2017 / Accepted: 29 March 2018 / Published online: 31 March 2018
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Abstract

Genotype I Japanese encephalitis virus (JEV) strain SCYA201201 was previously isolated from brain tissues of aborted piglets. In this study, we obtained an attenuated SCYA201201-0901 strain by serial passage of strain SCYA201201-1 in Syrian baby hamster kidney cells, combined with multiple plaque purifications and selection for virulence in mice. We investigated the genetic changes associated with attenuation by comparing the entire genomes of SCYA201201-0901 and SCYA201201-1. Sequence comparisons identified 14 common amino acid substitutions in the coding region, with two nucleotide point mutations in the 5'-untranslated region (UTR) and another three in the 3'-UTR, which differed between the attenuated and virulent strains. In addition, a total of 13 silent nucleotide mutations were found after attenuation. These substitutions, alone or in combination, may be responsible for the attenuated phenotype of the SCYA201201-0901 strain in mice. This information will contribute to our understanding of attenuation and of the molecular basis of virulence in genotype I strains such as SCYA201201-0901, as well as aiding the development of safer JEV vaccines.

Keywords Japanese encephalitis virus · Virulence attenuation · Genetic analysis · Mutation site · Adaptation

Introduction

The mosquito-borne Japanese encephalitis virus (JEV) belongs to the genus *Flavivirus*, family *Flaviviridae*, which also includes other clinically relevant arthropod-borne viruses, such as Yellow fever virus, West Nile virus, Tick-borne encephalitis virus, Dengue virus, and Zika virus. JEV is a leading cause of encephalitis in eastern and southern Asia [1], and its incidence is expected to increase in

Bangladesh, Cambodia, Indonesia, Laos, Myanmar, North Korea, and Pakistan [2]. There are approximately 67,900 cases of Japanese encephalitis annually, including about 33,900 (50%) in China (excluding Taiwan) [3]. Patients with Japanese encephalitis typically present after a few days with a non-specific febrile illness, followed by headache, vomiting, and reduced consciousness, often heralded by a convulsion [4]. Pigs are the reservoir hosts of JEV, and thus play a critical role in the transmission of the virus between mosquitoes and humans. Furthermore, JEV has been linked to breeding disorders in pigs and to severe economic losses in the swine industry in China [5].

JEV contains a 5' untranslated regions (UTR) followed by a 10,296-nucleotide (nt) coding region, which encodes three structural proteins (C, prM, and E), seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5), and a 3'-UTR. Phylogenetic analysis of the nucleotide sequences of the C/PrM and E genes allows characterization of the viruses, and showed that JEVs can generally be classified into five genotypes [4]. The most protective vaccine to date, known as SA14-14-2, was obtained from serial passage of JEV SA14 in primary hamster kidney cells, followed by several plaque purifications in primary chick embryo cells,

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Edited by Lorena Passarelli.

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peripheral passages in Syrian hamsters and suckling mice, and additional plaque purifications in PHK cells [6, 7]. The SA14-14-2 strain has been licensed in China, Nepal, India, Thailand, Sri Lanka, Cambodia, South Korea, and other Asian countries [8].

Three genotypes (I, III, V) have been confirmed to co-circulate in China in both high- and low-prevalence areas. Both genotype I and the Taiwan clade are newly introduced and have evolved relatively rapidly [9], and genotype III has been replaced by genotype I in South Korea, Thailand, and China [10]. However, little is known about the molecular mechanisms responsible for the attenuation of genotype I JEV strains. In this study, we obtained an attenuated strain SCYA201201-0901 from its virulent parental strain SCYA201201-1 and compared the entire genome of SCYA201201-0901 with those of SCYA201201-1, other genotype I strains, and the SA14-14-2 strain to identify the genomic changes related to the viral attenuation process.

Materials and methods

Viruses

The virulent JEV SCYA201201-1 strain was isolated from the brain tissues of aborted piglets in 2012 and maintained in our laboratory. SCYA201201-0901 was derived from the virulent parental SCYA201201-1 strain via consecutive passages in Syrian baby hamster kidney (BHK-21) cells, according to the attenuation processes shown in Table 1.

Mice

Three-week-old female BALB/c mice (purchased from Chengdu Institute of Biological Products, Chengdu, China) were maintained under controlled conditions (25 ± 1 °C, $40 \pm 10\%$ humidity) with free access to standard water and diet.

Passage of JEV in BHK-21 cells

BHK-21 cells (5×10^6) were infected with JEV derived from culture supernatants. The virus was allowed to adsorb for

1 h at 37 °C at a cell density of 5×10^6 /ml, and then diluted in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), to a density of 1×10^6 cells/ml. Cultures were incubated at 37 °C until a substantial cytopathic effect was observed, and virus derived from the culture supernatants was then stored in aliquots at -70 °C to allow for repeated culture, which should result in viral adaptation, as detected by subsequent analysis of viral RNA.

Plaque purification

BHK-21 cells were dispensed into six-well polystyrene plates (Costar, Cambridge, MA, USA) and incubated for 36 h at 37 °C until they formed a monolayer. Virus was allowed to adsorb as before and then diluted into 1% low gelling temperature agarose with 2% fetal bovine serum to a density of 1×10^6 cells/ml. Cultures were incubated at 37 °C until viral plaques were observed, and the plaques were then transferred to 500 μ l of fresh DMEM and used to infect BHK-21 cells as described above.

Virulence selection in mice

Groups ($n=6$ each) of 3-week-old female mice were inoculated intracerebrally with 30 μ l of 10^7 plaque-forming unit (PFU) dilutions of the plaque-purified virus. The mortality rate of each group was recorded over 21 days. The 50% lethal dose (LD50) of the virus was calculated using the Reed–Muench method, and the plaque-purified virus with the lowest LD50 was selected for further passage.

Mouse challenge experiments

Sixty 3-week-old female mice were divided into 10 groups ($n=6$ each). Seven of the groups were inoculated intracerebrally with 30 μ l of 10-fold serial dilutions of SCYA201201-1, and the other three groups were inoculated similarly with SCYA201201-0901. The mortality rate of each group was recorded for 21 days and the LD50 of the virus was calculated using the Reed–Muench method.

Table 1 Attenuation history of Japanese encephalitis SCYA201201-0901 strain

Methods	Names
Parental JEV SCYA201201-1 virus isolated from the brain tissues of aborted piglets	SCYA201201-1
16 passages of SCYA201201-1 in BHK-21 cells	SCYA201201
86 passages in BHK-21 cells and 3 plaque purifications of small plaque variant of SCYA2012-1	SCYA201201-86
120 passages in BHK-21 cells and 3 plaque purifications with virulence selections of 3-week-old mice inoculated intracranially	SCYA201201-0901

Detection of viral RNA replication in mouse brains

Twelve 3-week-old female mice were divided into two groups ($n=6$ each). One group was inoculated intracerebrally with 30 μ l of 2000 PFU of SCYA201201-1 and the other was similarly inoculated with SCYA201201-0901. Brain tissues were collected from the infected mice at 3, 5, and 7 days post-infection (d.p.i) and titrated by standard quantitative reverse-transcription PCR (RT-PCR), as described previously [5].

Nucleotide sequencing

JEV RNA was extracted using a total RNA Mini Kit (TaKaRa Bio Inc., Shiga, Japan), and used immediately for cDNA synthesis. The full-length virus genomes were PCR-amplified using eight gene-specific primer pairs, as described previously [5], using high-fidelity DNA polymerase. The sequences of the 5'- and 3'-UTRs were obtained using the 5' and 3' RACE Systems for Rapid Amplification of cDNA Ends kits (Invitrogen Corporation, Carlsbad, CA, USA). The PCR products of the JEV nucleotide sequences were cloned and sequenced using Sanger sequencing by TSINGKI Biotech (Chengdu, China) and Sangon Biotech (Shanghai, China). The genomic sequences were sequenced twice by each of the companies.

Sequence analysis

The complete nucleotide and amino acid sequences of the attenuated strain SCYA201201-0901 were compared with its virulent parental strain SCYA201201-1. One attenuated Mie/41/2002 (GenBank Accession No. AB241119), two virulent genotype I JEV strains HEN0701 (GenBank Accession No. FJ495189) and SX09S-01 (GenBank Accession No. HQ893545), and one vaccine strain SA14-14-2 (GenBank Accession No. AF315119) were used in the analysis. These were selected because Mie/41/2002, HEN0701, and SX09S-01 have known virulence characteristics and belong to genotype I, while SA14-14-2 is currently used as a JEV vaccine strain. A phylogenetic tree was constructed using MEGA5.05 software based on the E gene nucleotide sequences of the SCYA201201-0901, SCYA201201-1, and 23 known JEV strains of different genotypes.

Results

Virulence differences between SCYA201201-0901 and SCYA201201-1

The virulences of SCYA201201-0901 and SCYA201201-1 were investigated in mice. Inoculation with 10 PFU of

SCYA201201-1 caused 3-week-old mice to die of encephalitis, while injection with 10^7 PFU of SCYA201201-0901 caused no clinical signs (Fig. 1).

Differences in viral RNA replication between SCYA201201-0901 and SCYA201201-1 in mouse brains

Strain SCYA201201-1 could be detected in mouse brain tissue at 3 d.p.i., peaking at 7 d.p.i., while SCYA201201-0901 levels peaked at 5 d.p.i., and showed a significantly lower viral titer than SCYA201201-1 (Fig. 2).

Genomic organization of SCYA201201-0901 and SCYA201201-1 strains and phylogenetic analysis

The genome sequences of SCYA201201-0901 and SCYA201201-1 were determined to be 10,965 nt in length and contained a 96-nt 5'-UTR, a 10,296-nt open reading frame encoding a polyprotein of 3432 amino acids, and a 573-nt 3'-UTR. The genome sequences of SCYA201201-0901 and SCYA201201-1 were deposited in the GenBank database under the accession numbers KU508408 and MF124315, respectively.

A neighbor-joining tree based on the nucleotide sequences of the E gene indicated that SCYA201201-1 and SCYA201201-0901 were grouped with other genotype I strains (Fig. 3). Strain SCYA201201-0901 had 99.66% nucleotide and 99.56% amino acid identities with strain SCYA201201-1.

Amount of virus (PFU)	SCYA201201-0901	SCYA201201-1
	3 week old	3 week old
10^7	0/6 ^a	ND ^b
10^6	0/6	ND
10^5	0/6	6/6
10^4	ND	6/6
10^3	ND	6/6
10^2	ND	6/6
10^1	ND	3/6
10^0	ND	0/6
10^{-1}	ND	0/6

^a Number of mortal mice/number of mice inoculated with virus.

^b Experiment was not done.

Fig. 1 Mouse neurovirulence tests from infection through the intracerebral route. The mortality was monitored for 21 days after inoculation

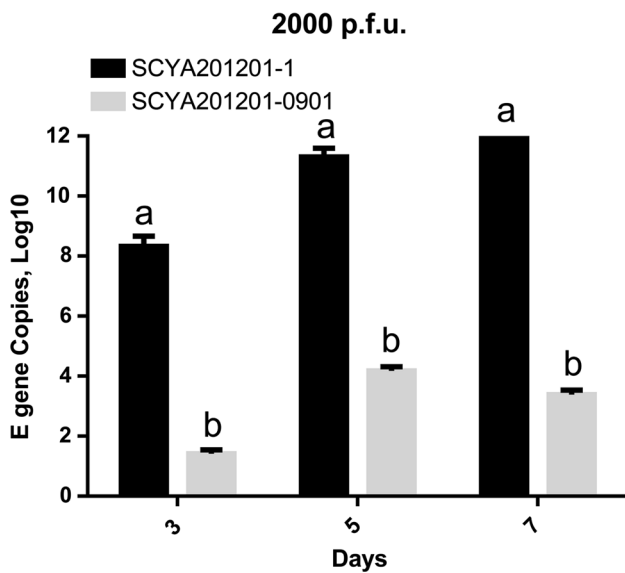


Fig. 2 Viral RNA replication tests in the brains of mice. Mouse brain tissues from the neurovirulence tests were harvested separately, homogenized, and titrated by standard quantitative RT-PCR (expressed in log₁₀ units). Data are the mean \pm SD, with different letters indicating a significant difference between groups (a or b) ($p < 0.05$)

Comparison of UTR sequences among different JEV strains

The nucleotide sequences of SCYA201201-0901, SCYA201201-1, other genotype I JEV strains, and SA14-14-2 were compared. Differences in the 5'-UTR between the attenuated SCYA201201-0901 and virulent SCYA201201-1 strains were limited, with only two nucleotide mutations (Table 2), while the 3'-UTR region included three nucleotide mutations between the attenuated SCYA201201-0901 and SCYA201201-1 strains (Table 2).

Comparison of JEV protein coding regions

Comparison of the protein coding regions among attenuated SCYA201201-0901, SCYA201201-1, other genotype I JEV strains and SA14-14-2 revealed no deletion/insertion mutations. A total of 14 amino acid substitutions occurred during the process of attenuation of the SCYA201201-1 strain, scattered across two structural proteins (M and E) and four non-structural proteins (NS1, NS2B, NS4A, and NS5), and all those substitutions were strongly conserved among genotype I strains. There were no amino acid changes in the C, NS2A, NS3, or NS4B proteins following attenuation (Table 3). In addition, a total of 13 characteristic nucleotide substitutions, all producing silent mutations, were found in seven genes (M, E, NS1, NS2B, NS3, NS4A, NS5) (Table 4).

Discussion

Genotype I JEV strains are currently emerging and spreading in China, and a better understanding of the molecular mechanisms responsible for attenuation of these strains will help to identify novel therapeutics and aid the development of safer vaccines for JEV. Furthermore, although genotype III JEV is presently the predominant genotype in pigs, genotype I has emerged and spread extensively in recent years [11], and further studies of these strains in pigs are thus becoming increasingly important. We obtained an attenuated SCYA201201-0901 strain by serial passage of strain SCYA201201-1 in BHK-21 cells, combined with multiple plaque purifications and virulence selection in mice. The LD₅₀ of strain SCYA201201-1 in 3-week-old mice was only 10 PFU, compared with $> 10^7$ PFU for SCYA201201-0901, indicating that the SCYA201201-0901 strain was highly attenuated. SCYA201201-0901 and SCYA201201-1 showed different pathogenicities in adult mice, and these two JEV strains thus provide an excellent experimental model for investigating the molecular mechanisms of genotype I JEV attenuation.

The attenuated SCYA201201-0901 and virulent SCYA201201-1 strains were shown to be closely related to each other genetically, with only 32 nucleotide differences in the entire 10,965-nt long genome, suggesting that these substitutions, alone or in combination, may be responsible for SCYA201201-0901 attenuation. The 5'-UTR was previously demonstrated to be important for West Nile virus RNA synthesis and virus replication [12]. There were only two nucleotide differences in the 5'-UTR between SCYA201201-0901 and SCYA201201-1, located at positions 43 and 52. A52-G in SCYA201201-0901 was identical to that in the virulent SX09S-01, suggesting that this mutation was unlikely to be involved in the process of attenuation. The G43-A mutation was highly conserved among four genotype I strains. Genomic RNA terminates with the conserved dinucleotides 5'-AG and CU-3' [13, 14], the 3' stem-loop structure [13, 15], and the short 5' and 3' cyclization sequences [16], which are the three major conserved elements required for flavivirus RNA replication. Sequence analysis showed that the G43-A mutation was not included in any of these three elements, indicating that this mutation may also not likely to play a critical role in the attenuation of SCYA201201-0901. There were three nucleotide differences in the 3'-UTR between the attenuated SCYA201201-0901 and virulent SCYA201201-1 strains, with point mutations at position 13 in the V domain, position 271 in the CS3 of the I domain, and position 324 in the II-1 domain. However, a JEV mutant lacking domains V, X, I, and II-1 was able to replicate in hamster BHK-21 and human neuroblastoma SH-SY5Y cells [17], suggesting that none of these three

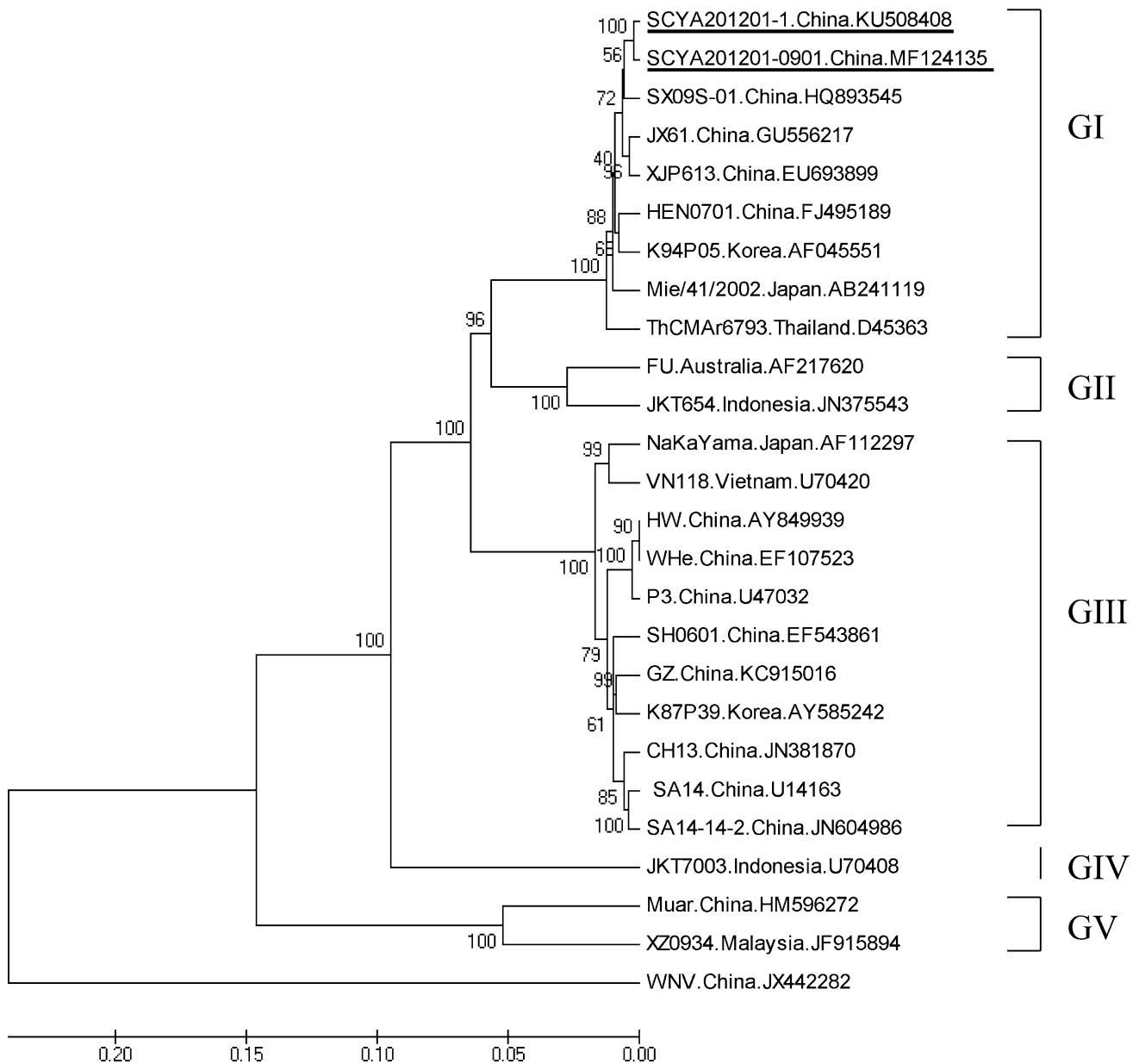


Fig. 3 Phylogenetic analysis of the JEV genome sequences. Phylogenetic trees constructed by the neighbor-joining method in MEGA 5.05, using nucleotide sequences of the E gene. The trees are drawn to scale, with branch lengths proportional to the number of substi-

tutions per site. The sequences determined in the present study are underlined. The sequence of a West Nile virus (WNV) isolate is used as an outgroup to root the tree

Table 2 Comparison of nucleotide mutations in untranslated regions between SCYA201201-0901, SCYA201201-1, and other genotype I JEV strains

Region	Position ^a	SCYA201201-0901	SCYA201201-1	Mie/41/2002	HEN0701	SX09S-01	SA14-14-2
5'-UTR	43	G	A	A	A	A	A
5T-UTR	52	A	G	G	0	A	0
3'-UTR	13	U	C	C	C	C	C
3'-UTR	271	U	C	C	C	C	C
3'-UTR	324	G	A	A	A	A	A

^aNucleotide position corresponds to the nucleotide sequence of the SCYA201201-0901

Table 3 Amino acid differences in the protein coding regions between the virulent and attenuated genotype I JEV strains

Region	Position ^a	SCYA201201-0901	SCYA201201-1	Mie141/2002	HEN0701	SX09S-01	SA14-14-2
prM	86	R	H	H	H	H	H
	109	ID	E	E	E	E	E
	72	T	A	A	A	A	A
E	176	R	I	I	I	I	V
	251	Y	S	S	S	S	S
	273	K	E	E	E	E	E
NS1	100	T	A	A	A	A	A
	269	D	N	D	D	D	D
NS2B	44	A	V	V	V	V	V
	96	T	A	A	A	A	A
NS4A	168	E	G	G	G	G	G
	248	L	F	F	F	F	F
NS5	623	I	V	V	V	V	V
	879	T	I	I	I	I	I

^aAmino acid position corresponds to the nucleotide sequence of the SCYA201201-0901

Table 4 Synonymous nucleotide substitutions in the protein coding regions between the virulent and attenuated genotype I JEV strains

Region	Position ^a	SCYA201201-0901	SCYA201201-1	Mie/41/2002	HEN0701	SX 09S-01	SA14-14-2
prM	540	GAT	GAC	GAC	GAT	GAC	GAC
	930	CAG	CAA	CAA	CAA	CAA	CAA
E	1065	AGT	AGC	AGC	AGC	AGC	AGC
NS1	3594	ACA	ACC	ACC	ACC	ACC	ACC
	3642	GCA	GCG	GCG	GCG	GCG	GCG
NS2B	4452	TTA	TTG	TTG	TTG	TTG	CTG
	5325	CTT	CTC	CTC	CTC	CTC	CTC
NS3	5541	GCA	GCG	GCA	GCA	GCA	GCA
	6417	GCA	GCG	GCA	GCA	GCA	GCA
NS4A	6912	GCA	GCG	GCA	GCA	GCA	GCA
	8505	CAA	CAG	CAG	CAG	CAG	CAG
N S5	9243	CGT	CGG	CGT	CGT	CGT	CGT
	9747	TCT	TCG	TCG	TCG	TCG	TCA

^aNucleotide position corresponds to the nucleotide sequence of the SCYA201201-0901

mutations in the 3'-UTR was likely to be related to attenuation of the SCYA201201-0901 strain.

Two substitutions (H86R, E109D) in the structural prM protein region were observed during the attenuation process. Flavivirus prM protein plays a crucial role in conformational folding of the E protein and protects it against premature acidification during the maturation process [18]. Tyrosine 78 has been reported to be a critical determinant present in the prM protein ectodomain and required for correct flavivirus assembly [19]. Single substitution of S138 N in the prM protein of Zika virus led to increased mortality in neonatal mice [20]. Additionally, amino acids 20–38 downstream from the prM cleavage site of Dengue virus modulated prM cleavage,

maturation of particles, and virus entry [21]. We therefore concluded that these two mutations might be involved in the process of virus attenuation.

The highest mutation rate occurred in the E protein, with four amino acid substitutions during the attenuation process. It was previously proposed that changes in the viral surface may play an important role in attenuation, and a series of mutations in the E protein were identified as the primary genetic determinant of attenuation of the JEV SA14-14-2 vaccine strain [22]. The E protein is therefore likely to play an important role in the attenuation process. Sequence analysis showed that three of the mutations (A72T, S251Y, and E273K) were in domain II of the E protein, while I176R was

in domain I. Domain I has previously been reported to participate in the E protein structural rearrangements required for fusion, while domain II encodes the fusion loop involved in pH-dependent fusion of the virus with the host cell membrane [6, 23]. In addition, two of the mutations (A72T, S251Y) included amino acids with similar natures, while two others (I176R, E273K) resulted in changes of amino acid charge. Mutation of some envelope protein amino acids close to GAG-binding domains to basic amino acids was suggested to affect virulence in Dengue virus [24]. A single substitution of E138K in domain I of JEV strain AT31 significantly attenuated the virus in mice [25], and the T380R mutation in the E protein of Yellow fever virus reduced its virulence in mice [26]. Mutating E176 in combination with E177 and other mutations by reverse genetic engineering also influenced virus virulence [27]. We therefore suspect that these four substitutions are likely to be related to the attenuation of SCYA201201-0901 in mice.

Two substitutions (A100T, D269N) occurred in the JEV genomic NS1 region during the viral attenuation process. NS1 is a multifunctional protein involved in virus replication and assembly [28], and also in the regulation of the host immune response [29]. The virulent strains HEN0701 and SX09S-01 also had a residue N269D substitution, suggesting that this is unlikely to be essential for attenuation. Furthermore, given that threonine and alanine residues share the same amino acid properties, we assume that the substitution A100T may also not play an important role in attenuation. A V44A substitution was observed during the attenuation process in the NS2B protein, and the same substitution in TMD2 of NS2B was associated with slight-to-moderate defects in JEV viral replication [30]. We therefore concluded that this substitution might play a role in the attenuation process by reducing viral replication. Three substitutions (A96T, G168E, F248L) were observed in the non-structural protein NS4A region after attenuation. Flavivirus NS4A is regarded as a central ‘organizer’ and is an important membrane protein for viral replication [31–33], and a previous study reported that a single Lys-to-Arg mutation at position 79 in NS4A significantly impaired JEV replication [34]. The A96T and F248L substitutions included amino acids with similar natures, and were thus less likely to be key determinants of attenuation, while G168E may play a role in SCYA201201-0901 attenuation by affecting viral replication. V623I and I879T substitutions were also observed in the NS5 protein, which is required for viral replication and 5′ capping [35]. However, both these substitutions included amino acids with similar natures and we therefore assumed that neither the V623I nor I879T substitution was likely to play a critical role in the attenuation of the SCYA201201-0901 strain.

In conclusion, we obtained an attenuated genotype I JEV strain SCYA201201-0901 and compared its full nucleotide

and amino acid sequences with those of its virulent parental strain SCYA201201-1, other genotype I strains, and the SA14-14-2 strain. We identified genomic changes in the protein coding and untranslated regions, and discussed these changes in relation to virus attenuation in the context of current knowledge of the molecular biology of JEV. We identified various candidate amino acid substitutions that might play roles in the attenuation phenotype of the SCYA201201-0901 strain. However, further studies are needed to determine which of these mutations are directly involved in and the mechanisms responsible for the attenuation process in SCYA201201-1. We are currently constructing a set of mutant recombinant JEVs by reverse genetic engineering to confirm the determinants of virulence during the attenuation process.

Acknowledgments This work was supported by the National Program on Key Research Project of China (2016YFD0500403). We thank lady Qingjun Li for helpful advice.

Author contributions RW and SJC conceived and designed the study. QZ, XTW, and YPW collected the samples. YF, QGY, YH, XPM, and YYZ carried out the experiments. XFH and YYZ drafted the manuscript.

Compliance with ethical standards

Conflicts of interest Authors declare that there were no conflict of interests.

Research involving human participants and/or animals All procedures performed in the present study involving animals were approved by the Institutional Animal Care and Use Committee of Sichuan Agriculture University (Approval Number BK2014-047), Sichuan, China and followed the guidelines of the National Institutes of Health.

Informed consent All authors read and approved the final manuscript.

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