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RESEARCH ARTICLE

A natural variation in the RNA polymerase of severe fever with thrombocytopenia syndrome virus enhances viral replication and in vivo virulence

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Abstract

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging tick-borne disease with high mortality in Eastern Asia. The disease is caused by the SFTS virus (SFTSV), also known as *Dabie bandavirus*, which has a segmented RNA genome consisting of L, M, and S segments. Previous studies have suggested differential viral virulence depending on the genotypes of SFTSV; however, the critical viral factor involved in the differential viral virulence is unknown. Here, we found a significant difference in viral replication in vitro and virulence in vivo between two Korean isolates belonging to the F and B genotypes, respectively. By generating viral reassortants using the two viral strains, we demonstrated that the L segment, which encodes viral RNA-dependent RNA polymerase (RdRp), is responsible for the enhanced viral replication and virulence. Comparison of amino acid sequences and viral replication rates revealed a point variation, E251K, on the surface of RdRp to be the most significant determinant for the enhanced viral replication rate and in vivo virulence. The effect of the variation was further confirmed using recombinant

Kyeongseok Jeon and Hyo-Jin Ro contributed equally to this work.

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SFTSV generated by reverse genetic engineering. Therefore, our results indicate that natural variations affecting the viral replicase activity could significantly contribute to the viral virulence of SFTSV.

KEYWORDS

genotype, reverse genetics, RNA-dependent RNA polymerase, severe fever with thrombocytopenia syndrome virus, viral replication, virulence

1 | INTRODUCTION

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging tick-borne disease caused by the SFTS virus (SFTSV), officially named *Dabie bandavirus*, which belongs to the genus *Banyangvirus* in the family Phenuiviridae.¹ SFTS is associated with symptoms including high fever, thrombocytopenia, leukocytopenia, myalgia, gastrointestinal illness, and neurological manifestations and has been reported in China, Japan, South Korea, and recently in Taiwan and Thailand.^{1–5} This tick-borne infection may also be prevalent in southern Asian countries, including Vietnam, Myanmar, and Pakistan.^{6–8} The cumulative number of SFTS cases has rapidly increased, and the case fatality rate is highly variable, ranging from 10% to 30%, depending on patients' age, sex, and endemic regions.^{9–11} However, the reason for the variable mortality rates remains unknown.

SFTSV possesses segmented RNA genomes, L, M, and S. The L segment encodes the RNA-dependent RNA polymerase (RdRp) involved in viral RNA replication,¹² while the M segment encodes the surface glycoproteins, Gn and Gc, associated with viral entry into host cells and the primary targets of neutralizing antibodies.^{13,14} The S segment encodes a nucleoprotein (NP) and nonstructural S segment protein that modulates the host innate immune system.^{15,16} Based on the phylogenetic analysis of viral genomes, SFTSV can be subdivided into several clades or genotypes, presenting genetic heterogeneity with uneven geographic distribution in endemic areas (Supporting Information: Table S1).^{17–20} The genetic diversity of SFTSV has been further generated through recombination and reassortment events and their spreading, potentially through bird migration.^{18,21}

Recently, several studies have revealed a potential association of viral clades/genotypes with clinical outcomes of SFTS in animal infection models and human patients.^{19,20} However, the molecular details in genomic variations associated with viral virulence and/or disease outcomes have not been well defined, even though it is well known that persistent high viral loads during acute phase of infection have been significantly associated with clinical severity and lethal outcomes.^{22,23} In this study, we examined the relationship between viral genomic variations and both in vitro and in vivo replication rates, as well as in vivo virulence. We conducted this analysis by generating genetic reassortants from two clinical isolates, which displayed significant differences in viral replication rates and in vivo virulence. Furthermore, we defined specific natural variations associated with enhanced viral replication and virulence using recombinant SFTSV generated by reverse genetic engineering. This approach enabled us

to identify the specific role of RdRp polymorphisms in viral replication and virulence and to provide novel insight into the pathogenic diversity derived from genetic variations in the viral polymerase of SFTSV.

2 | MATERIALS AND METHODS

2.1 | Study approval

This study received approval from the Institutional Animal Care and Use Committee of Seoul National University and Korea Zoonosis Research Institute (SNU-200512-5-4; JBNU 2020-0142). Animal experiments were conducted at the Korea Zoonosis Research Institute in an Animal Biosafety Level 3 facility, and were performed in strict accordance with the recommendations outlined in the National Guide Line for the Care and Use of Laboratory Animals.

2.2 | Genetic characterization and phylogenetic analysis

Genetic and phylogenetic analyses were conducted by aligning published full-length sequences of SFTSV reported from China, Japan, and South Korea until 2020 (obtained from GenBank, <https://www.ncbi.nlm.nih.gov/>). The analyses included a total of 476 full-length sequences of the L segment, 526 of the M segment, and 715 of the S segment (Supporting Information: Table S2–S4). Genomic sequences of the L, M, and S segments were aligned using MAFFT (v7.310)²⁴ and manually trimmed in AliView (v1.28). Phylogenetic relationships of the L, M, and S sequences were then reconstructed using IQTree (v2.2.2.6)²⁵ by setting of a best-fit nucleotide substitution model (L gene, GTR+F+R4; M gene, GTR+F+R3; and S gene, TVMe+R3) with 1000 replicates bootstrap approximation for the assessment of branch supports. Visualization of each tree was done in FigTree (v1.4.4), and bootstrap scores of tree branches were indicated in the phylogenetic trees.

2.3 | Preparation of SFTSV

The wild-type SFTSV strains, 2015-JJ01 (JJ strain, NCBI Accession number: MN329148-329140) and Gangwon/Korea/2012 (GW strain,

NCBI Accession number: KF358691-358693)³ were isolated from patients in South Korea. The KACNH3 (NCBI accession number: KP663743-663745), KAJNH2 (KU507547, KU507552, and KU507557), KASJH (KP663731-663733), KAGNH (KU507545, KU507550, and KU507555), and KADGH4 (KU507544, KU507549, and KU507554) strains were provided by the National Culture Collection for Pathogens in South Korea. All virus strains used in this study were propagated in Vero E6 cells (ATCC CRL-1586). The supernatant of infected cells was harvested at 4 days after infection and stored at -80°C after filtering with a $0.45\text{ }\mu\text{m}$ syringe filter. The infectious viral titer was determined by a focus-forming assay and presented as a focus-forming unit (FFU).¹⁴ Briefly, the filtered supernatant was serially diluted and added to a monolayer of Vero E6 cells, and the cells were incubated at 37°C for 90 min. The viral supernatant was then removed, and the cells were incubated under overlay media (DMEM supplemented with 10% FBS and 0.8% methylcellulose) at 37°C for 7 days. Cells were fixed with 4% paraformaldehyde (Intron) and permeabilized with 0.2% Triton X-100 (Sigma). The SFTSV foci were detected using rabbit anti-SFTS NP antibody (Abclon) and goat anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (Invitrogen). Viral plaques were visualized by incubation with a 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) solution containing 0.01% hydrogen peroxide (Sigma-Aldrich), and their images were collected using a microscope (Olympus IX-51; Olympus); then plaque sizes were measured using ImageJ (National Institutes of Health).

2.4 | Quantitative reverse transcript-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from SFTSV-infected cells, culture supernatants, and mouse spleens using Trizol or Trizol LS Reagent (Life Technologies) according to the manufacturer's instructions. RNA was reverse-transcribed into cDNA using the HiSenScript RH (–) RT Premix kit (Intron). The cDNA was quantified using the SensiFAST Probe Lo-ROX kit (Bioline) with primers (forward: 5'-CCTTCAGGTCATGACAGCTGG-3', reverse: 5'-ACCAGGCTCTCAATCACTCCTGT-3') and detecting probe (5'-6FAM-AGCACATGTC CAAGTGGGAAGGCTCTG-BHQ1-3') derived from the NP gene of SFTSV. The qRT-PCR was performed on the BioRad CFX connect real-time system (Bio-Rad).

2.5 | Animal experiment

Female Interferon α/β receptor knockout (IFNAR KO, C57BL/6) mice aged 6–12 weeks were used for infection tests.¹⁴ They were housed and maintained in a specific pathogen-free facility at Seoul National University College of Medicine. The mice were transferred to the Korea Zoonosis Research Institute in an Animal Biosafety Level 3 facility for infection study, subcutaneously injected with 100 FFU of each SFTSV strain, and their body weight and survival were monitored daily. At 3 days postinfection, spleens and blood were

collected from the mice and used for qRT-PCR and hematological analysis, respectively. The platelet counts were analyzed using a hematology analyzer BC-2800 (Mindray).

2.6 | Generation of reassortant viruses

A monolayer of Vero E6 cells was coinfecting with both GW and JJ strains at a multiplicity of infection of 10 for each virus simultaneously. The viral inoculum was added to the cellular monolayer in a T-75 flask and incubated at 37°C for 90 min. After washing, the cells were cultured in 10 mL of DMEM with 2% FBS and incubated at 37°C with 5% CO_2 for 4 days. Following incubation, the supernatant was serially diluted and transferred to a healthy Vero E6 monolayer for plaque assay. At 9 days postinfection, well-separated SFTSV plaques were picked with a pipette and transferred to a microfuge tube containing 100 μL of serum-free DMEM, then propagated in Vero E6 cells. The isolated viral clones were amplified using a RT-PCR kit (Accupower Taq PCR PreMix; Bioneer) with specific primer sets targeting L, M, and S segments (Supporting Information: Table S5) for sequence analysis. Representative sequencing results of the reassortant viruses without any contamination are presented in Supporting Information: Figure S1.

2.7 | Construction of mutant SFTSV using reverse genetics system

The reverse genetics system of SFTSV²⁶ was kindly provided by Professor Jae U. Jung at Lerner Research Institute of Cleveland Clinic and Dr. Benjamin Brennan at Medical Research Council-University of Glasgow Centre for Virus Research. To perform site-directed mutagenesis of selected amino acid residues, the Phusion Site-Directed Mutagenesis Kit (Thermo Fisher Scientific) was used with pTVT7-HB29ppL5 as a DNA template. A pair of designed primers (Supporting Information: Table S6) was used for each amino acid substitution. Sanger sequencing (Macrogen) was conducted to confirm the presence of desired variations in all plasmids. Recombinant viruses were generated by transfecting five plasmids to BSR-T7/5 cells, as previously described.²⁶ Briefly, 7.5×10^5 BSR-T7/5 cells were transfected with 0.1 μg pTM1-HB29ppL, 0.5 μg pTM1-HB29N, 1 μg pTVT7-HB29S, 1 μg pTVT7-HB29M, and 1 μg pTVT7-HB29ppL using 3 $\mu\text{L}/\mu\text{g}$ of DNA of TransIT-LT1 (Mirus Bio LLC) as a transfection reagent. After 5 days of transfection, virus-containing supernatants were collected, amplified in Vero E6 cells, and stored at -80°C until use.

2.8 | Statistical analysis

The data was analyzed with nonlinear regression, *t*-test, one-way ANOVA, or Log-rank (Mantel–Cox) test for survival rate embedded in Graph Pad Prism 8 software (GraphPad Software).

2.9 | Structural analysis of RdRp

The structure of L protein (Protein Data Bank [PDB] ID: 8ASD) was adopted from previous studies.^{27,28} Graphical representation of the protein structure, as well as structural analysis and coulombic electrostatic surface potential calculation were performed with UCSF ChimeraX (Release Candidate version 1.6).²⁹

3 | RESULTS

3.1 | Genomic diversity of SFTSV and their local distribution in the endemic region

To assess the genetic diversity of SFTSV, we collected 476 L, 526 M, and 715 S segment sequences deposited in NCBI GenBank before January 2021. Segment-based phylogenetic analysis using the L, M, and S nucleotide sequences revealed seven major viral clades (Figure 1A), including A–F, as reported previously.^{17,20,30} Complete genomes comprising L, M, and S sequences were available for 471 viral strains, and 19 strains (4.0%) possessed reassorted genomes (Supporting Information: Table S7). Among the various genotype clades, clade B was the most common genotype, accounting for 45.8% based on L segment sequences and was highly prevalent in South Korea and Japan (Figure 1B). Clade F was the second most dominant genotype, occupying 27.3%, and mainly reported in China. Clade A was also prevalent in China, accounting for 13.0%, but was a minor one in South Korea and Japan. This obvious region-related pattern was consistently observed in previous studies.^{17,20} In South Korea, all 17 complete genome sequences isolated in Jeju Island belong to clade B, and only two complete viral genomes have genotype D and F reported in Inland South Korea (Figure 2A). Taken together, the genomic diversity of SFTSV is highly variable in China, while limited variations have been detected in South Korea and Japan.

3.2 | Variation in viral replication rate and in vivo virulence of SFTSV clinical isolates

Next, we investigated the impact of genetic diversity on viral replication rate and in vivo virulence. We selected two different clinical isolates, 2015-JJ01 (JJ strain from Jeju island) and Gangwon/Korea/2012 (GW strain from Gangwon province in Inland South Korea) for comparative analysis since these two types belong to the major B clade and minor F clade, respectively, in South Korea. The average size of viral foci ($0.31 \pm 0.09 \text{ mm}^2$) formed in Vero E6 cell monolayers by the GW strain was significantly larger than those formed by the JJ strain ($0.14 \pm 0.05 \text{ mm}^2$) (Figure 2B,C), indicating more rapid viral replication and/or spreading of the GW strain than that of the JJ strain in the host cells. Consistently, infection of IFNAR KO mice with the GW strain caused more severe disease when compared to the mice challenged with the same dose (100 FFU/mouse) of the JJ strain, as assessed by survival rate, loss of body

weight, and the progression of thrombocytopenia (Figure 2D–F). The difference in in vivo virulence might be due to viral replication rate since the viral load was significantly higher, approximately 19 times, in the spleens of mice infected with the GW strain (mean: 1.9×10^7 copies/mg of spleen) than in those infected with the JJ strain (mean: 1.0×10^6 copies/mg of spleen) at 3 days postinfection (Figure 2G).

3.3 | The L segment encoding RdRp is a determinant of enhanced viral growth and virulence

To identify the viral factor causing the difference in replication rate and virulence, we generated various reassortant viruses derived from coinfection of GW and JJ strains, resulting in six types of reassortant viruses (R1–R6). The viral genomes of R1, R2, and R3 are composed of JJ genomes with replaced L, M, or S segments derived from the GW strain, respectively, while R4, R5, and R6 have GW strain-based genomes with substituted L, M, or S segments from the JJ strain (Figure 3A). Cells were infected with each reassortant and the size of foci was measured at 7 days postinfection (Figure 3B). Cells infected with R1 showed significantly larger foci than those infected with JJ, but the size of foci formed by R2 and R3 were similar to those of the JJ strain. In contrast, R4 and R5 generated smaller foci than GW, but R6 formed a similar size of foci to the GW strain. Moreover, when analyzing all foci formed by the wild type and reassortants possessing a common genome segment, the average size of foci presented significant differences only between the virus groups containing different L segments (Figure 3C). GW, R1, R5, and R6, which possessed the L segment from the GW strain, formed significantly larger foci than JJ, R2, R3, and R4, whereas the viral groups containing different M or S segments derived from GW or JJ did not generate significant differences in the size of foci (Figure 3B,C). These results indicate that the SFTSV L segment encoding RdRp activity is the primary factor determining the enhanced viral replication rate in vitro. Indeed, when we assessed kinetic changes of viral RNA levels in infected cells and culture supernatants (Figure 3D,E), the copy number of viral RNAs in cells infected with R1 or GW, possessing the L segment derived from the GW strain, increased more rapidly before 60 h postinfection when compared to those with JJ or R4 containing the L segment from JJ strain (Figure 3D). In addition, more viral RNAs were released, potentially representing the levels of progeny viruses, from the cells infected with R1 or GW than in cells infected with JJ or R4 (Figure 3E). Therefore, the L segment derived from the GW strain belonging to F clade supports more rapid viral replication when compared to that of the JJ strain.

To further evaluate the effect of different viral L segments on in vivo virulence of SFTSV, IFNAR KO mice were challenged with 100 FFU of JJ, R1, GW, or R4, then the survival rate, body weight change, and viral load in the spleen were monitored. Consistent with viral growth in vitro cellular infection, we observed mice infected with R1 or GW strains more rapidly succumbed to death (Figure 4A,D), presented faster body weight loss (Figure 4B,E), and showed more enhanced viral loads in spleens (Figure 4C,F) when compared to those challenged with JJ or R4 strains. Taken together, the L segment derived from the GW strain

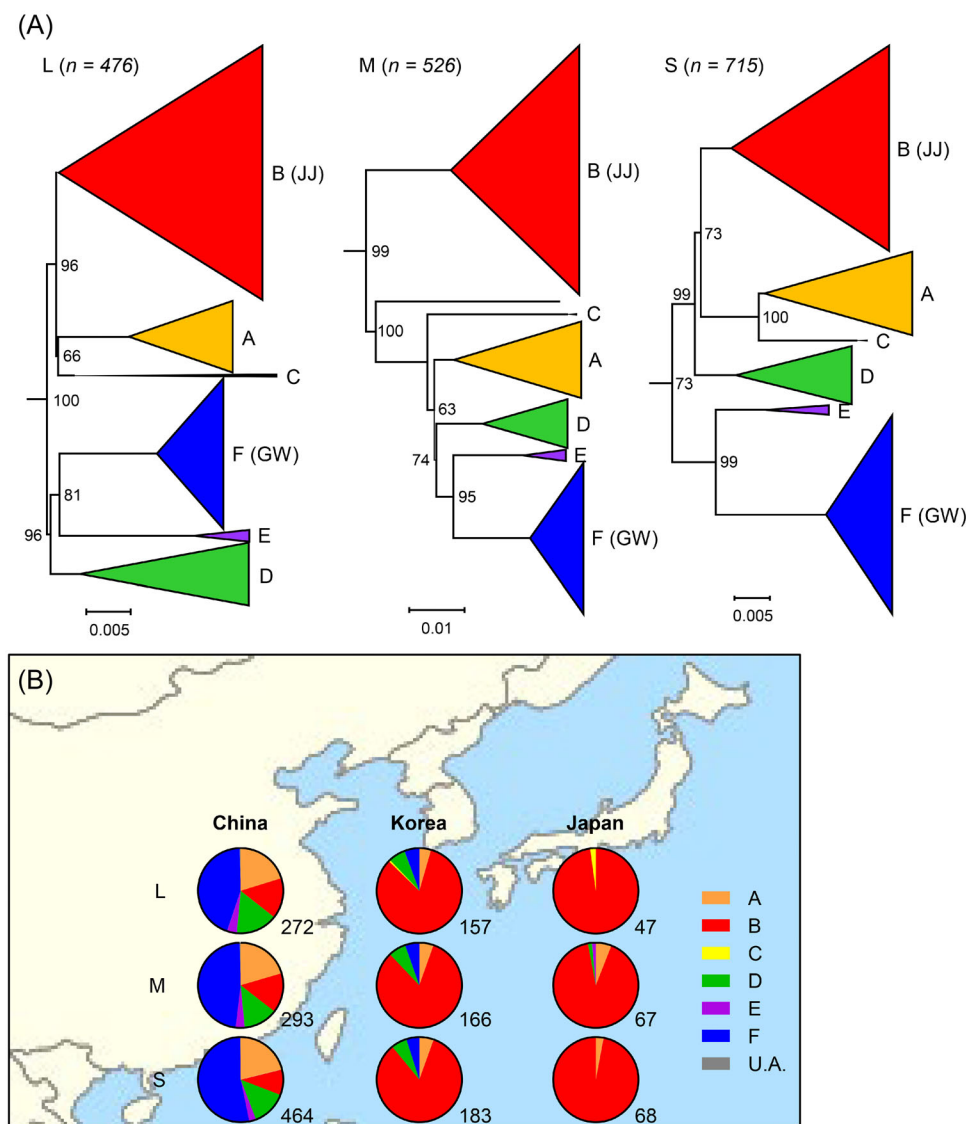


FIGURE 1 Phylogenetic analysis of SFTSV genome sequence isolated from China, Korea, and Japan. (A) phylogenetic analysis based on the full-length ORF sequences of L ($n = 476$), M ($n = 526$), and S ($n = 715$) of SFTSV sequences. The bootstrap values resulting from 1000 replications are shown at each branch, and a scale bar indicates nucleotide substitution. The SFTSV clades A, B, C, D, E, and F are color-coded as orange, red, yellow, green, purple, and blue, respectively. (B) The proportion of SFTSV genetic clades reported from China, Korea, and Japan. The numbers next to the graphs are the number of sequences used for analysis. SFTSV, severe fever with thrombocytopenia syndrome virus.

can support more increased viral replication, thereby enhancing viral virulence in vivo, when compared to that of the JJ strain. These results are also further suggesting that differential viral RdRp activity might be the primary determinant for the increased viral replication.

3.4 | Identification of RdRp variation associated with increased viral replication

To further define the viral sequence variations in RdRp responsible for enhanced viral replication, we measured and compared the size of viral foci in cells infected with eight available SFTSV clones. The GW strain belonging to F clade showed the largest foci reproducibly compared to

other strains of B and D clades (Figure 5A). When we investigated the amino acid variations observed in the eight viral strains, we found 10 variable amino acid sequences within RdRp proteins (Figure 5B). Among these, four amino acid variations (E251K, A470T, E1061D, and I1711V) were specifically present in the GW strain. Based on the previous structural analysis of RdRp,^{12,31} the E251K variation is located in a linker region, A407T in an influenza PA-C-like domain, E1061D in the RdRp core domain, and I1711V in the cap-binding domain (CBD) (Figure 5B, upper diagram). We focused on E251K and A407T variations because of their significant changes in charge (E251K) or polarity (A407T). In addition, we selected the A1433T substitution observed in GW strain, as well as in KASJH strain belonging to the D clade, for further study because it was recently determined to be associated with

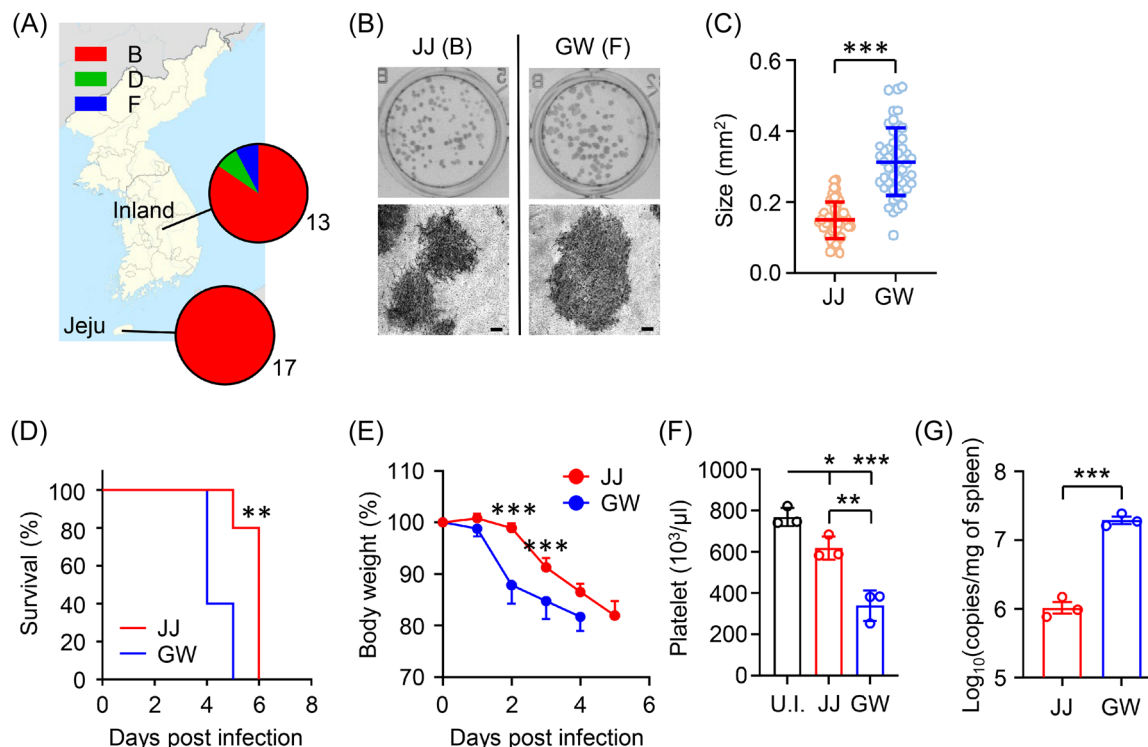


FIGURE 2 Comparison of viral growth and virulence between SFTSV GW and JJ Strains. (A) The proportion of SFTSV genotypes in South Korea. The genomic sequences of SFTSV isolated from SFTS patients in mainland or Jeju Island are used for analysis. (B) The image shows foci generated by infection with SFTSV JJ or GW strain representing the B and F clades, respectively. Scale bar = 100 μ m. (C) Comparison of the viral foci size ($n = 50$). It was measured using ImageJ software and the difference was analyzed by Student t -test. (D) Survival rate ($n = 5$ /group), (E) body weight changes ($n = 5$ /group), (F) the number of blood platelets at 3 days after infection ($n = 3$ /group), and (G) viral loads in spleen ($n = 3$ /group) of IFNAR KO mice infected with 100 FFU SFTSV, JJ, and GW strain. Black: uninfected (U.I.); red, JJ-infected; blue, GW-infected. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ analyzed by Mann-Whitney test for pairwise comparison and one-way ANOVA for multiple comparisons (Kruskal-Wallis test). FFU, focus-forming unit; SFTSV, severe fever with thrombocytopenia syndrome virus.

SFTS fatality.²⁰ Comparison of viral foci in cells infected with GW strain containing both 251K and 407T variations with those from all other seven viral strains possessing 251E and 470A revealed a statistically significant enhancement of viral growth of GW strain (Figure 5C). The overall size of viral foci in cells infected with GW and KASJH strains retaining 1433T variation is also significantly larger than that of viral foci from other strains carrying 1433A. Therefore, we decided to confirm the effect of these point variations on viral replication and virulence.

3.5 | The E251K variation on the surface of RdRp enhances replication rate and virulence of SFTSV

To investigate the effect of specific substitutions in the RdRp protein, we performed site-specific mutagenesis of the target amino acid residues, E251K, A470T, or A1433T, using the pTVT7-HB29ppl plasmid encoding SFTSV HB29 L segment via a reverse-genetic engineering system.²⁶ We generated three types of recombinant viruses, in which the HB29 wild-type genome was substituted with E251K, E251K/A470T, or A1433T in the L segment, and assessed their growth rate after infecting Vero E6 cells. The mutant virus carrying the E251K variation presented significantly larger viral foci

than wild type or a mutant including A1433T variation did (Figure 6A). Another viral mutant possessing both E251K and A470T variations also showed a similar level of foci size, comparable to that of the virus carrying the E251K variation alone, suggesting a minor role of the A470T variation. Even though the A1433T variation induced significantly larger foci than the wild-type virus did, its growth-promoting effect was significantly lower than that of the E251K variation. In addition, viral RNA amplification in cells infected with a recombinant HB29 clone carrying the E251K variation (Figure 6B), as well as the amount of viral RNA released into the culture supernatant (Figure 6C), was generally higher than those of mutant viruses including the A1433T variation or wild-type virus. Consistently, IFNAR KO mice infected with the mutant virus carrying the E251K variation more rapidly succumbed to death than those infected with the same amount of wild-type virus or recombinant virus including the A1433T variation (Figure 6D). In addition, the E251K mutant virus also showed significantly higher viral loads in spleens (Figure 6E) and lower platelet counts in blood (Figure 6F) at 3 days after infection when compared to mice infected with wild-type virus. The virus carrying the A1433T variation presented an intermediate level of disease severity as assessed by survival rate, viral loads, and blood platelet counts in comparison with the

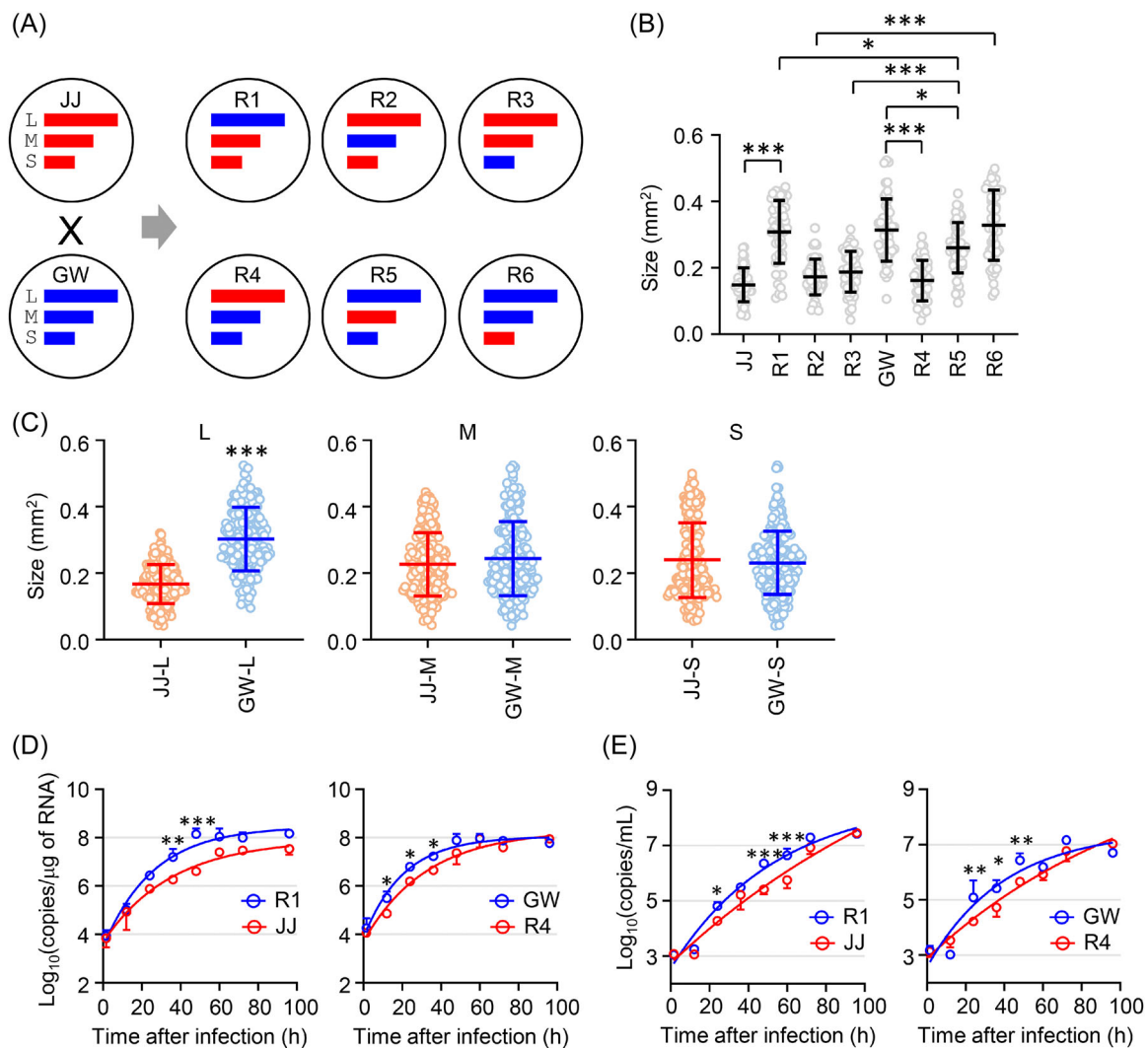


FIGURE 3 Enhanced viral growth induced by L segment derived from SFTSV GW strain. (A) The types of recombinant viruses formed by reassortment of segmented viral genomes after coculturing JJ and GW strains. (B) The size of foci ($n = 50/\text{group}$) formed by the recombinant virus infection was compared with that of the wild-type viruses JJ and GW strain. (C) Comparison of the focus size ($n = 200/\text{group}$) after reclassifying based on origin of L, M, and S segmented genomes. Red: derived from JJ strain and blue: derived from GW strain. (D) Kinetic changes of the viral RNA counts in Vero E6 cells over time after infection (triplicate experiments). Left panel; R1 (blue) or JJ strain (red), right panel; GW strain (blue) or R4 (red). (E) Kinetic changes of the viral RNA counts in the supernatant of culture medium (triplicate experiments). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ analyzed by Mann-Whitney test for pairwise comparison and one-way ANOVA for multiple comparisons (Kruskal-Wallis test). SFTSV, severe fever with thrombocytopenia syndrome virus.

wild-type and E251K mutant virus. These results clearly indicate that the E251K variation in the RdRp of SFTSV not only induces more rapid viral replication in vitro and in vivo but also causes a more virulent phenotype during in vivo infection, potentially by promoting viral growth rate.

4 | DISCUSSION

Even though the critical determinant for persistent viral replication in severe SFTS patients has not been clearly defined, previous studies have consistently reported that a persistent high viral load in SFTS patients during the acute phase of infection is one of the most

significant predictors of fatality and severe disease.^{9,22,23} Both inefficient host defense against viral infection and superior viral replication supporting enhanced viral virulence could be associated with sustained higher viral load, thereby resulting in critical outcomes. In a recent study using a large scale of clinical data and viral genomic information, viral sequence variation was significantly associated with clinical outcomes and disease fatality.²⁰ They showed that a specific viral clade IV, equivalent to previously defined clade D,¹⁹ was associated with a significantly increased case fatality, pronounced inflammatory response featured by more production of various inflammatory cytokines, and significantly elevated viremia measured at 5–8 days postdisease onset.²⁰ Furthermore, a total of 25 missense variations (five in the NS, nine in the Gn, four in the Gc, and

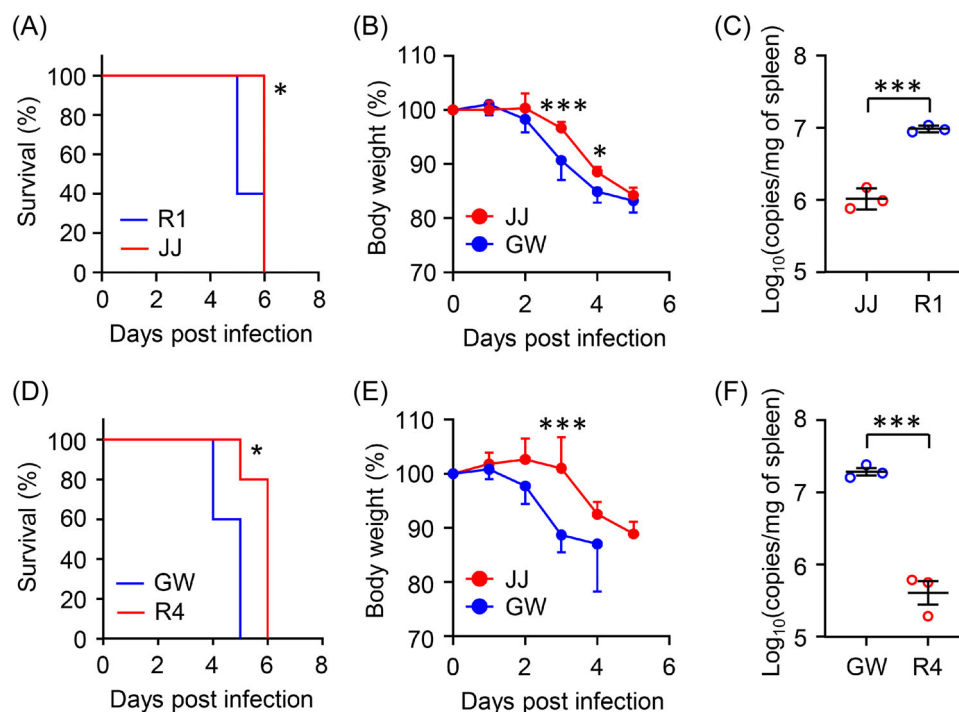


FIGURE 4 Enhanced in vivo virulence induced by L segment from SFTSV GW strain. IFNAR KO mice were challenged subcutaneously with 100 FFU of SFTSV, (A–C) R1 or JJ, (D–F) GW or R4. Survival rates (A, D; $n = 5/\text{group}$), body weight changes (B, E; $n = 5/\text{group}$) and viral loads in spleens (C, F; $n = 3/\text{group}$) are presented. (A–C) Red: R1 infected and blue; JJ infected. (D–F) Red; GW infected and blue; R4 infected. * $p < 0.05$, *** $p < 0.001$ analyzed by Mann–Whitney test. FFU, focus-forming unit; SFTSV, severe fever with thrombocytopenia syndrome virus.

seven in the RdRp) were associated with SFTS fatality using multivariate regression analysis after adjusting for sex and hospitalization delay.²⁰ The seven missense variations in RdRp associated with increased mortality are Y566F, I651V, R835K, S1038T, T1433A, I1447V, and R1684K. Among them, R1684K showed the highest odds ratio for disease fatality. However, SFTSV strains representing the viral clades did not show an apparent difference in replication rate in Vero cell culture up to 72 h after infection.²⁰ In addition, no significant difference in viral titers was observed in spleen samples from a lethal C57BL/6 mouse model, which was pretreated with an anti-IFNAR1 IgG antibody, after infection with various strains representing the viral clades.²⁰ Even though this is an interesting approach to provide significant insight into primary virulent factors by investigating specific associations of viral genomic variation with clinical outcomes and the degree of inflammation in human patients, the complex impact of viral sequence heterogeneity in different viral factors and clinical variations may hardly define the specific role of each viral factor and/or their natural variations in viral replication rate and in vivo virulence.

In this study, we clearly demonstrated that the L segment encoding RdRp is the most significant factor determining viral replication rate among the three segmented genomic RNAs by comparing two clinical isolates that presented differential in vitro and in vivo replication rates. This is the first report showing the relative contribution of each viral RNA segment of SFTSV to viral replication, both in vitro and in vivo infection systems, using genetic reassortant

viruses. Since we used Vero E6 cells, which have lost the ability to produce type I IFN due to spontaneous gene deletions,³² and IFNAR KO mice³³ for SFTSV infection, the replication rate of SFTSV in these infection models might depend primarily on the viral replication capability, minimizing the potential inhibitory effect of the host innate defense mechanism during the acute phase of infection. We found two variations, E251K and A1433T, that significantly enhanced viral replication in Vero E6 cells and viral virulence in IFNAR KO mice using recombinant viruses carrying the specific variations generated by the reverse genetics system (Figure 6). In addition, we confirmed that an SFTSV reassortant, possessing the L segment with K251 and T1433 variations from the GW strain, along with M/S segments from the KADGH4 strain (which exhibits the smallest foci, as shown in Figure 5), forms foci that are significantly larger than those of the KADGH4 strain (Supporting Information: Figure S2). The foci sizes of this reassortant are also similar to those of the GW strain. The SFTSV GW strain with K251 and T1433 of RdRp was isolated from a lethal human infection.³ However, clinical information on a SFTS patient from whom JJ strain with E251 and A1433 of RdRp was isolated is unavailable. Further clinical analysis needs to be conducted to determine whether disease severity is associated with the viral genotypes carrying the natural variations in RdRp.

When we analyzed the sequence differences in the noncoding regions of SFTS genomes, we observed only a few variations in the 5' and 3' untranslated regions (UTRs) of L, M, and S segments, as well as in the intergenic region of the S segment (Supporting

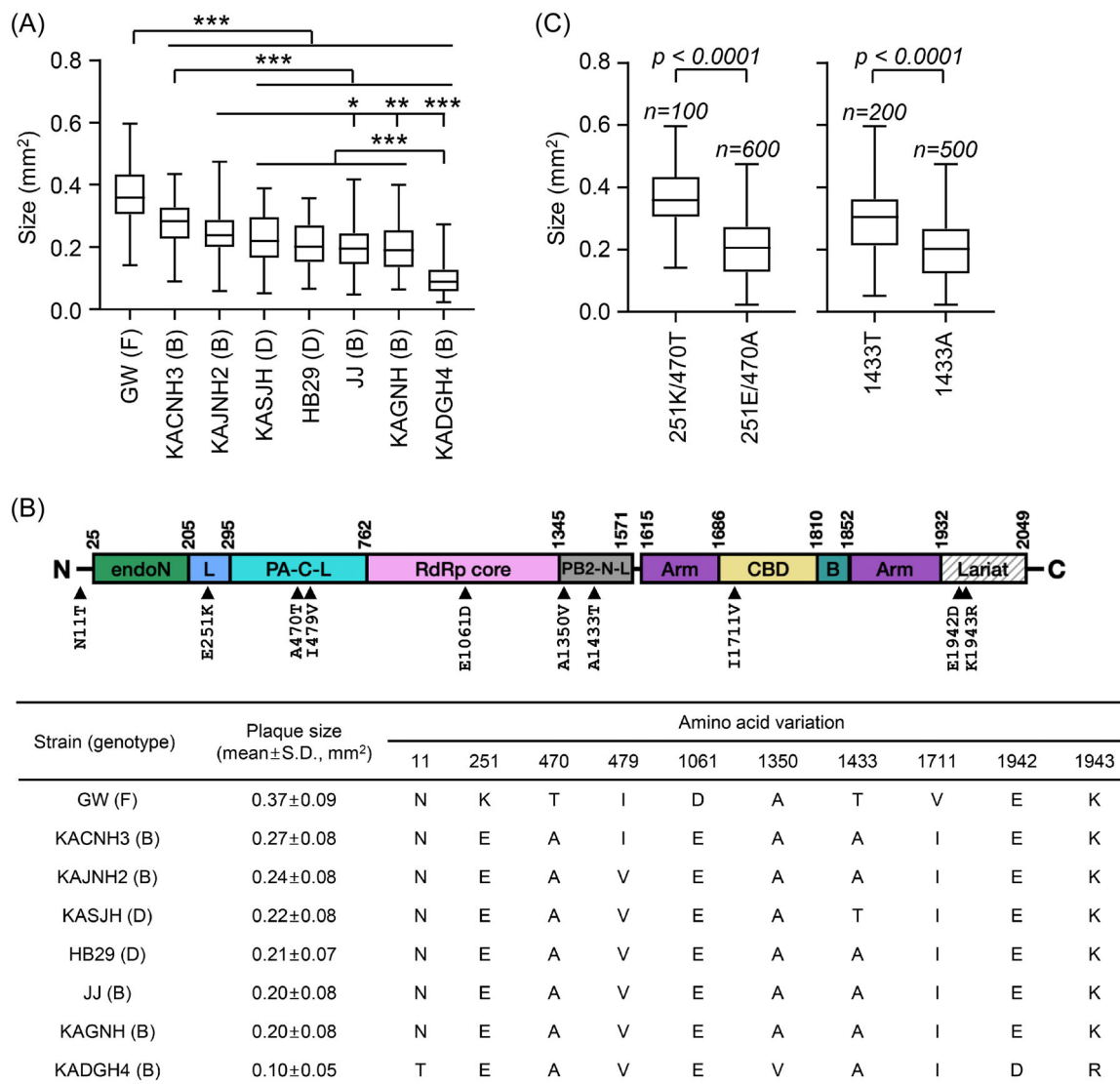


FIGURE 5 Comparison of viral growth among various SFTSV strains belonging to diverse genetic clades and their sequence variation in RdRp protein. (A) Comparison of foci size generated by several virus strains belonging to B clade (JJ, KACNH3, KAJNH2, KAGNH, and KADGH4), D clade (rHB29 and KASJH), and F clade (GW). The size of foci ($n = 92$ –100/group) is measured by ImageJ. Box plots present median, minimum, and maximum values. (B) Diagram of RdRp protein domains and location of nonsynonymous variations detected in the SFTSV strains. List of nonsynonymous variations observed in the displayed SFTSV strains and their average foci sizes was summarized (lower table). (C) Comparison of foci sizes ($n = 100$ –600/group as indicated) derived from viruses carrying indicated substitutions in RdRp protein. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ analyzed by Mann–Whitney test for pairwise comparison and one-way ANOVA for multiple comparisons (Kruskal–Wallis test). b, blocker motif; CBD, cap-binding domain; EndoN (upper panel), N-terminal endonuclease domain; L, linker region; PA-C-L, PA-C like domain; PB2-N-L, PB2-N like domain; RdRp core, RNA-dependent RNA polymerase; SFTSV, severe fever with thrombocytopenia syndrome virus.

Information: Figure S3). Considering that the sequence variations in the UTRs do not appear to significantly influence the secondary RNA structure, their impact on viral replication might be considered negligible.

Among the 476 RdRp sequences we used, the E251K variation was detected in 19 strains (4.0%) across various clades of SFTSV (3 in clade A, 8 in clade B, and 8 in clade F). This indicates that the variation is not clade-specific and is a minor natural variation. In addition, the E251K variation was detected in only 4 RdRp sequences out of the 805 additional sequence data reported from China

recently,²⁰ which confirms its rare prevalence in the endemic region. In contrast, the A1433T variation is relatively prevalent, occupying 50.6% (241/476) in our sequence data set. 1433T is also highly prevalent, occupying 90.4% (728/805) of the sequences, in the previous Chinese study.²⁰ Even though they found that 1433A is mainly detected in the most virulent clade IV (equivalent to our clade D) and is one of the significant missense variations linked to SFTS fatality (fatality rate of 1433T = 15.9% and 1433A = 32.0%), a specific association of the amino acid change with viral replication rate or in vivo virulence has not been validated. Therefore, our approach, which

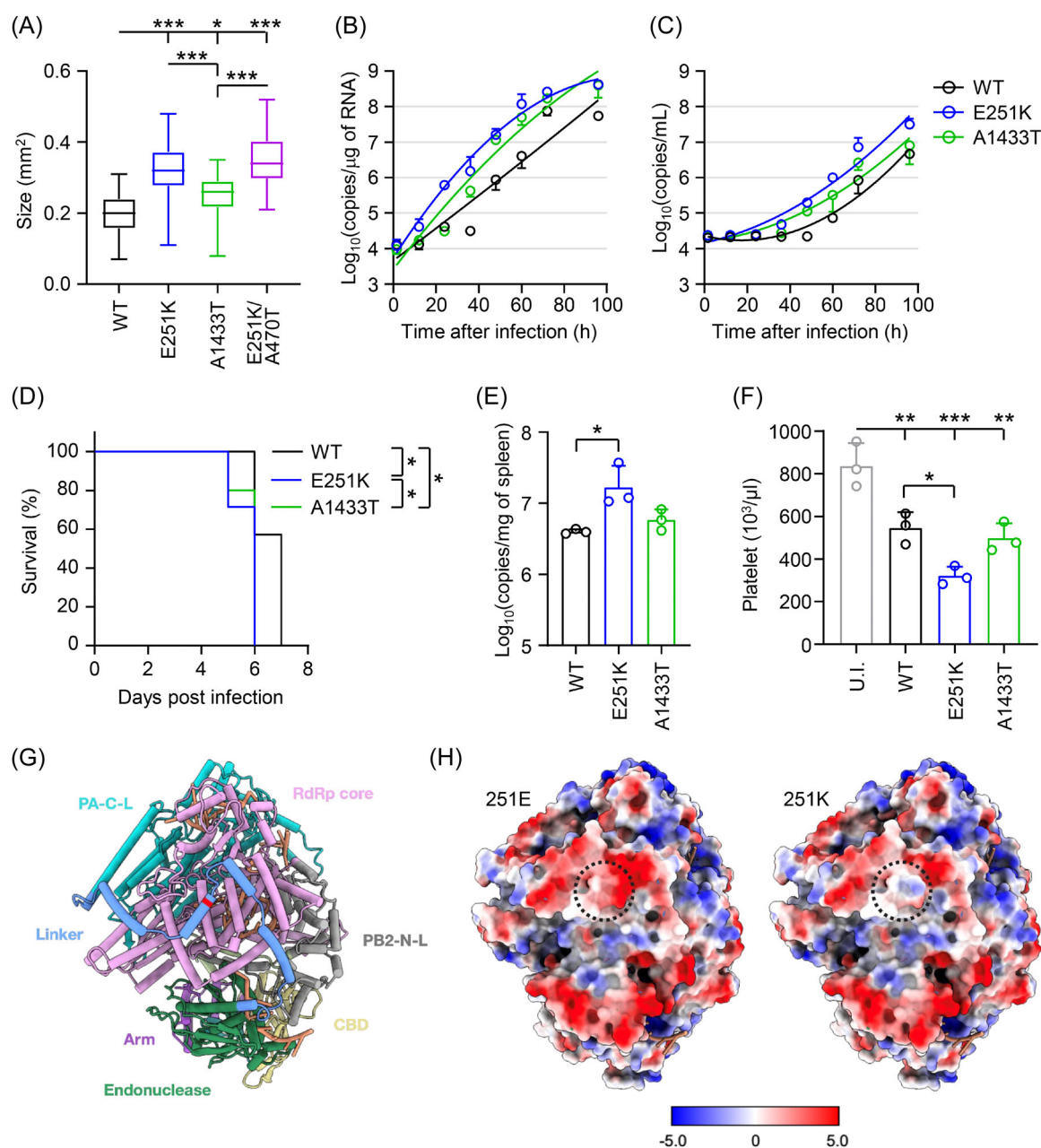


FIGURE 6 Enhanced viral replication rate and in vivo virulence induced by E251K substitution in RdRp of SFTSV. (A) Comparison of viral foci sizes ($n = 50/\text{group}$) generated by wild type (rHB29 strain) or mutant viruses carrying indicated substitutions in RdRp. Box plots present median, minimum, and maximum values. (B) Kinetic changes of the viral RNA counts in Vero E6 cells over time after infection with wild type or indicated mutant viruses (triplicate experiments). (C) Kinetic changes of the viral RNA counts in the supernatant of culture medium over time after infection with wild type or indicated mutant viruses (triplicate experiments). Survival rate (D, $n = 5$), viral loads in spleens (E, $n = 3$), and platelet counts in bloods (F, $n = 3$) of IFNAR KO mice infected with wild type or indicated mutant viruses are presented. Error bar: mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ analyzed by one-way ANOVA (Kruskal–Wallis test). (G) Location of E251K variation (red) on RdRp structure. The colors of proteins domains are coded as in Figure 5B. Orange: RNA molecules. (H) Electrostatic surface potential of linker region in RdRp structure. Left panel, electrostatic potential map of linker region in wild type. Right panel, electrostatic potential map of linker region with E251K substitution. Negative charges are shown in red and positive charges are shown in blue. The mutated region is indicated by dashed circle. RdRp, RNA-dependent RNA polymerase; SFTSV, severe fever with thrombocytopenia syndrome virus.

involves using recombinant SFTSV carrying specific point variations, may yield more convincing results regarding the molecular determinants of viral growth rate and virulence in the absence of competent type I IFN signaling. When we assessed the viral RNA replication in a

type I IFN-competent cell line (BJAB cells) that supported viral growth, however, we failed to observe significant differences (Supporting Information: Figure S4). The difference in replication rates of SFTSV may be abrogated and not necessarily associated with

enhanced virulence in the presence of competent innate immune responses upon infection.

In a previous study, Nada et al. reported that substitutions of an arginine residue (N1891) located in the arm domain of the C-terminal region with basic amino acids (K or R) enhanced polymerase activity, whereas substitutions with an acidic amino acid (E) decreased this activity.³⁴ However, this finding was not confirmed in viral mutants carrying the specific substitution, nor in in vivo infection models. Nonetheless, it has been speculated that the C-terminal domains of the SFTSV L protein play important roles in facilitating the transcription activity of the SFTSV RdRp protein, potentially involving cap-snatching activity.^{35,36} Meanwhile, E251K variation found in this study is located on the surface of linker region (Figure 6G,H). The linker, which connects the endonuclease to the influenza PA-C-like domain, wraps around the RdRp core and contributes to maintaining structural integrity and flexibility. While the endonuclease is required for priming the viral transcription via cap-snatching mechanism, the CBD of PB2-like subunit captures nascent host pre-mRNA.^{37,38} Conformational changes of both the linker and the endonuclease were suggested for the further elongation process.²⁸ The negatively charged surface of the linker region may be involved in interactions with other proteins or RNA molecules that are important for viral transcription initiation. Changes in the charge distribution on the linker surface resulting from the E251K variation (Figure 6H) could potentially affect these interactions, and our results indicate enhanced efficiency of viral RNA replication and/or transcription. A1433T is on thumb ring subdomain of PB2-N-like region and located near the RNA template exit channel (Supporting Information: Movie). The polarity change at this position may also affect the speed of RNA template exit process. These have to be further validated in the future study.

Taken together, multiple variations in the viral RdRp, even outside of catalytic core, have been generated naturally in the endemic region and may cause significant variation in viral replication rate, and thereby affecting the virulence of SFTSV. Notable contribution of L segment variation encoding RdRp on viral replication rate and in vivo virulence in comparison with other viral genes also highlights the potential impact of the point variations on wide variation of mortality and disease severity in endemic regions. Therefore, careful monitoring of the viral quasi-species, especially in RdRp gene, observed in clinical samples and wild tick hosts need to be continued for understating the relationships with the viral evolution, replication, and virulence in the accidental human host.

AUTHOR CONTRIBUTIONS

Kyeongseok Jeon: Conceptualization; investigation; resources; data curation; writing—original draft preparation. **Hyo-Jin Ro:** Conceptualization; investigation; resources; data curation. **Jun-Gu Kang:** Methodology; investigation; resources. project administration. funding acquisition. **Da-Eun Jeong:** Methodology; investigation. **Joowan Kim:** Methodology; investigation; resources. **Yebeen Lee:** Methodology; investigation; resources. **Ga-Yeon Yoon:** Methodology; investigation. **Ju-Il Kang:** investigation. **Joon-Yong Bae:** Methodology;

investigation. **Jin Il Kim:** Methodology; investigation. **Man-Seong Park:** Methodology; investigation; resources. **Keun Hwa Lee:** Resources. **Hyun-Soo Cho:** Methodology; investigation; writing—review and editing. **Yuri Kim:** Conceptualization; project administration; writing—review and editing. **Nam-Hyuk Cho:** Supervision; conceptualization; investigation; resources; writing—review and editing; project administration; funding acquisition. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data presented and supporting information in this study are available on request from the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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