Phylogenetic and Geographic Relationships of Severe Fever With Thrombocytopenia Syndrome Virus in China, South Korea, and Japan

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Background. Severe fever with thrombocytopenia syndrome (SFTS) is a tick-borne acute infectious disease caused by the SFTS virus (SFTSV). SFTS has been reported in China, South Korea, and Japan as a novel Bunyavirus. Although several molecular epidemiology and phylogenetic studies have been performed, the information obtained was limited, because the analyses included no or only a small number of SFTSV strains from Japan.

Methods. The nucleotide sequences of 75 SFTSV samples in Japan were newly determined directly from the patients’ serum samples. In addition, the sequences of 7 strains isolated in vitro were determined and compared with those in the patients’ serum samples. More than 90 strains that were identified in China, 1 strain in South Korea, and 50 strains in Japan were phylogenetically analyzed.

Results. The viruses were clustered into 2 clades, which were consistent with the geographic distribution. Three strains identified in Japan were clustered in the Chinese clade, and 4 strains identified in China and 26 in South Korea were clustered in the Japanese clade.

Conclusions. Two clades of SFTSV may have evolved separately over time. On rare occasions, the viruses were transmitted overseas to the region in which viruses of the other clade were prevalent.

Keywords. Bunyavirus; severe fever with thrombocytopenia syndrome; SFTS; SFTS virus; tick-borne virus infection.

Severe fever with thrombocytopenia syndrome (SFTS) is a tick-borne acute infectious disease with a high case mortality risk (approximately 6%–30%) [1–3]. The disease is caused by the SFTS virus (SFTSV), a novel Bunyavirus. As of March 2014, indigenous SFTS had been reported in several provinces/prefectures in China, Japan, and South Korea, where they are adjacent or separated by a sea [1, 3–6] (Figure 1A). The viral
A phylogenetic analysis of a viral genome is indispensable for understanding the features of genotypic variation, and the relationship of strains with their geographic distribution. It was previously demonstrated that 8 strains of SFTS recovered from patients in Japan were clustered to an independent lineage to which Chinese strains were clustered [3]. Because the previously reported analyses used no or only a very small number of Japanese SFTSV sequences [4–11], the findings do not provide
information about the evolutionary relationship of SFTSVs in Japan and China. Moreover, because the sequences in those studies may have been determined either directly from patient specimens or from in vitro isolates, the results may have been affected by nucleotide mutations that occurred in the isolates during the isolation process in vitro.

Hence, in the present study, the nucleotide sequences of 75 SFTSV samples in SFTS patients in Japan were determined directly from the serum samples, without performing a viral isolation process in vitro. In addition, the entire sequences of 7 strains isolated in Vero cells in vitro were also determined and compared with those determined directly from the patients’ serum samples. The phylogenetic analysis suggested that the 2 clades of SFTSV strains evolved separately over time and were rarely transmitted to the other regions.

MATERIALS AND METHODS

Serum Samples From SFTSV-infected Patients
Medical personnel in Japan were requested to inform us on a voluntary basis if they treated patients with symptoms similar to those of SFTS from 30th January 2013 to 30th June 2014 [3]. Thanks to the courtesy of the prefectural and municipal public health institutes, the serum samples from 83 patients who were diagnosed to have SFTSV by SFTSV genome amplification using a reverse-transcriptase–polymerase chain reaction (RT-PCR) method from serum samples (as reported previously; [12]) were evaluated in this study. Of the 83 samples, 8 entire SFTSV genome sequences (YG1, SPL003, SPL004, SPL005, SPL010, SPL030, SPL032, and SPL035) were determined in the previous study [3]. It was noteworthy that none of the patients had any history of foreign travel before the onset of the disease.

SFTSV Isolation In Vitro
SFTSV were isolated from the patients’ serum samples by inoculation of the samples into Vero cells [3]. In brief, Vero cells were inoculated with the serum samples of RT-PCR-positive patients for virus isolation, and were cultured for 4 to 7 days. The propagation was examined with SFTSV antigen detection by an indirect immunofluorescence assay using a rabbit polyclonal antibody against SFTSV recombinant nucleoprotein [3]. The isolates from the patients named YG1, SPL003A, SPL004A, SPL005A, SPL010A, SPL030A, and SPL035A were employed in this study. All of the isolates were used within 2 passages in Vero cells.

Viral Genome Amplification
Total RNAs were extracted from 200 µL of each serum sample or viral culture supernatant using a High Pure Viral RNA Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer’s protocol. The elution volume for RNA extraction was 50 µL. The complementary DNAs (cDNAs) were synthesized from 10 µL of the extracted RNA solution using SuperScript III Reverse Transcriptase (Life Technologies, Carlsbad, California) with 250 ng of random primers at 50°C for 60 minutes according to the manufacturer’s protocol. SFTSV genome amplification by PCR covering the entire region was performed as follows. A total of 0.4 µL of cDNA solution was added to a PCR reaction mixture of QS Hotstart High-Fidelity 2X Master Mix (New England BioLabs, Ipswich, Massachusetts), which contained a 2 × reaction mix, H2O, and 0.4 µM of a specific primer set, as shown in Supplementary Table 1.

To ensure that the primers covered the entire viral genome, the first primer pairs were selected (Supplementary Table 1). If the primer sets did not work, the second to fifth choices of primer pairs, which included the region in the first primer sets, were used. After being denatured at 98°C for 30 seconds, the samples were subjected to 40–45 cycles of amplification under the following touch-down cycle conditions; denaturing at 98°C for 10 seconds, annealing temperature at 65°C for 30 seconds, and extension at 68°C for 20 seconds/kilo base pair (kb), followed by a 1°C decrease of the annealing temperature during the first 5 cycles and thereafter maintaining the annealing at 60°C for the rest of the time in a PCR machine (Eppendorf, Hamburg, Germany). The concentrations of PCR products were determined by electrophoresis on 1% agarose gels with 330 ng lane of 2-Log DNA Ladder (0.1–10.0 kb, NEB), which allowed the approximate amount of each band to be determined by staining with GelRed (Biotium, Hayward, California). The PCR products were combined into respective specimens of approximately equal molarity.

Whole-Genome Sequencing
The samples (ie, 75 out of 83 serum samples and 7 isolates) were sequenced using the next-generation sequencing technique, except for 30 of the S segments (ie, SPL130A to SPL172A), which were sequenced from the PCR products using the general Sanger sequencing technique with a 3500 × L Genetic Analyzer (Life Technologies), according to the manufacturer’s protocol.

For whole-genome sequencing using the next-generation sequencing technique, a total of 0.5 to 1 µg of all combined PCR products from a sample was fragmented to an average of 800 base pairs using the Covaris S220 focused ultrasonicator (Covaris, Woburn, Massachusetts). Following fragmentation, libraries were constructed using titanium chemistry according to the manufacturer’s instructions for Rapid Library Preparation with using GS Titanium Rapid Library MID Adaptors for distinguishing each of the samples and the emPCR Lib-L method (Roche Applied Science). The library was sequenced (a maximum of 10 samples in a sequence run) on a GS Junior sequencing system (Roche Applied Science) according to the manufacturer’s instructions. The sequencing data obtained were utilized to perform a reference-guided alignment with an
identified viral genome sequence using the GS Reference Mapper software program (Roche Applied Science) based on the default parameters. The nucleotide sequence variations were computed from the raw data aligned with the obtained sequence of each strain using the GS Reference Mapper with duplicate reads. To exclude any variations that were artificially generated from a deviation of the forward- and reverse-read variations, Fisher exact test was performed. The variation was rejected if the deviation was statistically significant (P < .05). The variations that were erroneously generated from the sequencing of homo-polynucleotide stretches were also rejected if there was a homo-polynucleotide stretch. Because the terminal sequences of the segments were conserved, the primer sequences were used for these in the terminal regions.

Phylogenetic Analysis
The nucleotide sequences determined in this study (ie, SPL053A to SPL172A) were deposited in the DDBJ/EMBL/GenBank databases. The accession numbers for these sequences and the others in the databases used in this analysis are listed in Supplementary Table 2. The nucleotide sequences of each segment used in this analysis were compiled as FASTA-formatted supplementary files (L segment: supplementary_data_L.txt, M segment: supplementary_data_M.txt, S segment: supplementary_data_S.txt). The sequence data of the viruses identified in China and South Korea were obtained from the database. As the criterion, the sequence data of each segment, which is more than 75% region of sequences in the segment determined, was selected from the database for phylogenetically analyses except for the SFTSV strains confirmed in South Korea. The sequence alignment was computed using the MUSCLE software program and the built-in MEGA6.1 software program [13]. The phylogenetic trees used to analyze the molecular evolution were constructed using the maximum likelihood method based on the Tamura–Nei model [14] using the MEGA program. The confidence of the tree was tested using 1000 bootstrap replications [15]. The codon positions included were the 1st + 2nd + 3rd + Noncoding. Initial tree(s) for the heuristic search were obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood approach. The “use all sites” option was chosen for the Gaps/Missing Data Treatment. The multiple sequence alignments and the phylogenetic trees of the S, M, and L segments of SFTSV are available in Treebase (http://purl.org/phylo/treebase/phylows/study/TB2:S17059).

Ethics Statement
The clinical specimens used for this study were collected after obtaining informed consent from the patients themselves (for those who survived) or their responsible family members (for those who died). All of the protocols and procedures were approved by the medical research ethics committee of the National Institute of Infectious Diseases for the use of human subjects (no. 506).

RESULTS
Sequence Determination of the SFTSV Strains Identified in Japan
The SFTSV genome was amplified from the patients’ serum samples by RT-PCR. The amplicons covered the entire segments of the genome by overlapping each of them. It was difficult to amplify a portion or entire segments of the viral genome from some of the serum samples that contained a small amount of the virus, as determined with the real-time RT-PCR method [12]. In 75 of the 83 patient serum samples (ie, excluding the 8 samples reported previously [3]), 58 entire and 17 partial S segments, 38 entire and 6 partial M segments, and 40 entire and 4 partial L segments of SFTSV were sequenced for the nucleotide sequence determination. In 3 cases, it was not possible to amplify the entire M and L segments.

Comparison of the Complete Genome Sequences of the Viruses in Patients’ Serum Samples and in the Clinical Isolates
The nucleotide sequence variations of 7 strains in the patients’ serum samples and their isolates in vitro were determined by

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<th>De novo Variations in Isolates</th>
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Abbreviation: SFTSV, severe fever with thrombocytopenia syndrome virus.
using a next-generation sequencing technique. The average coverage depth of each sample was ×500 to ×1200. There was a small degree of either synonymous or nonsynonymous variations present in the patients' serum samples (Table 1), but these variations did not seem to cluster in any specific region (Figure 2). The variations in the in vitro isolates are also indicated in Table 1 and Figure 2. A small degree of de novo variations following the isolation process was observed. However, similar to the variations in vivo, these variations did not form a cluster in any specific region (Figure 2), and most of the variations did not become dominant (ie, less than 50%). One isolate, YG1, did not possess any de novo nonsynonymous variations. The frequency of de novo variations in the isolates did not seem to be relevant to those passage numbers as long

Figure 2. The viral nucleotide variations in the patients' serum samples and in the in vitro isolates. The nucleotide variations in the L (A), M (B), and S (C) segments in the patients' serum samples from the YG1 (filled circle), SPL003A (filled square), SPL004A (filled triangle), SPL005A (filled reverse triangle), SPL010A (filled diamond), SPL030A (open circle), and SPL035A (open triangle) strains and in their in vitro isolates are plotted. The x-axis indicates the position of the nucleotide base, and the y-axis indicates the percentage of variation present in the nucleotide. The red symbol indicates that the nucleotide variation is a nonsynonymous mutation. The symbol enclosed by a square indicates that the nucleotide variation in the isolate was already present in the patient's serum. Abbreviations: GPC, glycoprotein precursor; L, large; M, medium; NP, nucleoprotein; NS, nonstructural protein; RdRp, RNA-dependent RNA polymerase; S, small.
Figure 3. The maximum likelihood trees of the SFTSV genome in radiation format for the L (A), M (B), and S (C) segments. The Heartland virus was used as an outgroup. The trees enclosed by red squares are magnified (D: L segment, E: M segment, F: S segment). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. In parts D, E, and F, the 2 clades are divided by color, in blue (Chinese) or yellow (Japanese). Seven of the genotypes are separated by enclosing them, and the names of genotypes (C1 to C5 and J1 to J3) are also shown. The strains that were identified from patients in Japan (the year of onset and the prefecture are shown in parentheses), China, or South Korea are indicated by red circles, blue squares, or a green triangle, respectively. The strains indicated by open symbols may have undergone segmental reassortment between genotypes. Abbreviations: L, large; M, medium; S, small; SFTSV, severe fever with thrombocytopenia syndrome virus.
Figure 4. The maximum likelihood trees of the SFTSV genome in rectangular format for the L (A), M (B), and S (C) segments. The details are described in Figure 3. The trees of the L and S segments only show the enlarged parts without the Heartland virus as an outgroup. The tree of the M segment did not include the Heartland virus for the analysis. The genotypes are shown to the right of the strains. The values of the bootstrap percentage are shown next to the branches. The strains marked by red circles, blue squares, or green triangles are those identified from patients in Japan, China, and South Korea, respectively. The strains indicated by open symbols may have undergone segmental reassortment between genotypes. For the strains identified in Japan, the year of onset and name of the prefecture where the patient was identified are shown in parentheses. Abbreviations: L, large; M, medium; S, small; SFTSV, severe fever with thrombocytopenia syndrome virus.
as the passage number was small (Table 1). Hence, the source of the sample (ie, patient’s serum specimen or in vitro isolate) does not appear to affect the results of the subsequent phylogenetic analysis.

**Phylogenetic Analysis**

The nucleotides sequences of the L, M, and S segments from 140 strains (87 from China, 1 from South Korea, and 52 from Japan), 171 strains (91 from China, 28 from South Korea, and 52 from Japan), and 211 strains (125 from China, 3 from South Korea, and 83 from Japan) strains, respectively, were phylogenetically analyzed using the corresponding segment of other Phleboviruses (ie, Heartland virus) as the outgroup (Figure 3). Only the maximum likelihood trees are indicated, but the neighbor-joining phylogenies were similar. The tree topology of the M segment was unreasonable if the tree construction was based on the partial sequences identified in South Korea, together with that of Heartland virus (data not shown). This was probably because the sequences were too short. Therefore, the outgroup comparison of the M segment was performed without including the sequences from South Korea. The outgroup comparison indicated that the root of SFTSV based on the M segment obviously divided the strains into 2 clades that were in accord with the strains identified in specific regions (ie, China or Japan; Figures 3B and 3E). Although the roots based on the L or S segment sequences did not clearly divide the strains in accord with those identified in the regions, the node that divided the samples into the 2 clades was still obvious and close to the root (Figures 3A, 3C, 3D and 3F). The clades were thus defined as Chinese or Japanese clades.

Moreover, it appeared that these 2 clades consisted of 8 genotypes (genotype C1 to C5 in China, J1 to J3 in Japan) (Figure 3D, 3E, and 3F). Although the L and M segments of SPL087A, SPL161A, and AHL/China/2011 were resolved into the Chinese clade based on the topological relationship to the root of SFTSV, these were not resolved into any specific genotypes. Therefore, these were resolved into genotype C5 for convenience (Figure 3D, 3E, and 3F). Except for genotypes C1 and C5, the genotypes in the Chinese clade were equally predominant. On the other hand, in the Japanese clade, genotype J1 was the major genotype, and genotypes J2 and J3 were the minor genotypes.

Three strains identified in Japan (ie, SPL087A, 153A, and 161A) were clustered into the Chinese clade (Figure 4), although none of the patients in Japan had a history of travel to China or South Korea before the onset of the disease. In addition, some strains identified in China (ie, ZLD, Zhao, Zhejiang/01/2011, zjzs02) were clustered into genotype J3 of the Japanese clade (Figures 3 and 4).

Most of the strains identified in South Korea were clustered into genotype J3 (Figures 3 and 4). Although the analysis based on the M segment sequences was performed without the Heartland virus, the results were also supported by at least 2 strains...
of SFTSV infection by isolation and/or SFTSV genome amplification of SFTSV strains between the patients (ie, AHL/China/2011, HZM, LN2012-14, -34, -41, -42, and -58) of the Japanese clade, but not in those of the Japanese clade (Figure 4).

DISCUSSION

Because SFTS is classified as a “notified infectious disease,” medical personnel whose patients are diagnosed to have SFTS must notify the Ministry of Health, Labour, and Welfare of Japan. Furthermore, we asked them to confirm the presence of SFTSV infection by isolation and/or SFTSV genome amplification with RT-PCR from the suspected patients’ serum samples. Almost all of the serum samples collected from such patients in Japan were sent to the Department of Virology I at the National Institute of Infectious Diseases. Hence, this study represents the molecular characteristics of all SFTSV strains currently circulating in Japan.

This is the first study to compare the sequence difference of SFTSV strains between the patients’ serum samples and the in vitro isolates. The nucleotide variations of SFTSV in the patients’ serum samples existed at a low frequency and at random in the genome. This suggests that no selective pressure, which might influence the occurrence of the variation in the patients, was present during the acute phase of the infection. Moreover, the SFTSV isolated using Vero cells basically retained their original nucleotide sequences. This indicates that SFTSV can be isolated without any of the adaptive mutations that occur due to the changes in the host factor requisites (eg, receptor specificity) and the ability to evade the immune system. These findings are highly informative not only for future phylogenetic analyses, but also for future in vitro and in vivo studies using SFTSV isolates.

The results of the phylogenetic analysis revealed that the phylogenetic path of SFTSV diverged from its phylogenetic root or the node that was close to the root to each of the Chinese and Japanese clades in view of the relationship between the clades and the geographical distribution, the SFTSV in the regions of China and Japan may have evolved independently from an early stage of emergence and maintained in each of these regions, which are geographically separated. One strain, KAGGT5, identified in South Korea, was clustered into the Japanese clades (Figure 4B). The sequence of KAGGT5 was typical of genotype J1. In addition, some of the strains (ie, SPL087A, 153A, 161A) identified in Japan were clustered into the Chinese clade, and vice versa (ie, ZLD, Zhejiang/01/2011, zjzs02, Zhao). These findings imply that some viruses in China and South Korea were transmitted, likely several times, from Japan across the East China Sea and/or the Sea of Japan, and vice versa. This fact was also supported by evidence that SFTS patients were confirmed in Jeju Island, South Korea [16], and there was no discernible prefectoral clustering of these strains in genotype J1, although the strains were distributed in the Honshu, Shikoku, and Kyusyu islands of Japan, which are separated by a sea. This information regarding the overseas transmission raises questions about what the transporter for SFTSV is. On a global basis, migratory birds have been reported to be a transporter of ticks infected with various human pathogens, such as *Borrelia, Rickettsia*, and Crimean-Congo hemorrhagic fever virus (CCHFV), a member of *Bunyaviridae* [17–21]. However, at present, there are other possibilities, such as the transport of humans and/or livestock accompanied by traveling and trade, because the cultural exchanges between China, South Korea, and Japan have been active for many centuries. Further investigations, such as viral gene detection from the ticks in the area where the specific strains were identified and from those on migratory birds, is needed to obtain more detailed information.

In a comparison between the Chinese and Japanese clades, the M segment was the most phylogenetically diverse compared with the other segments. It is known that the CCHFV M segment also contains greater genetic diversity compared with the others [22]. Because the M segment encodes Gn and Gc glycoproteins, which are responsible for binding to cell-surface receptors, the diversity is likely to be a result of the adaptation to infect local ticks and vertebrate hosts. Likewise, the segmental reassortment might have occurred for adaptation when a strain was introduced into a new geographic region. The SPL087A, 161A, and AHL/China/2011 L and M segments were provisionally clustered into genotype C5, because these were difficult to cluster into the other genotypes. In addition, the segments of SPL087 and 161A may have undergone reassortment, but there is no definitive evidence for this, because these L and M segments were difficult to cluster into a typical genotype, and the S segments were clustered into genotype C4, but were atypical of the genotype. This may have been the result of accumulating nucleotide mutations; the S segments showed segmental reassortment between the genotypes, and these may have also been the result of adaptation to the regional ticks and vertebrates.

Most of the strains identified in South Korea were in genotype J3, which is a minor genotype in Japan, although this study indicates that both Chinese and Japanese clades of
SFTSV strains are widespread in South Korea [23]. It is thought that the genotype J3 was clustered into the Japanese clade on the basis of the phylogenetic analyses. However, geographically, genotype J3 may be mainly endemic in South Korea, and that may be the reason why the strains of genotype J3 were the most frequently observed strains in the Japanese clade in the regions of China and South Korea. The strains of genotype J3 existing in China were mainly identified in Zhejiang province, which has been deemed to be a nonepidemic region in China [9, 10, 24]. In addition, it was possible that the strains clustered into genotypes J2 and J3 showed regional clustering in Hyogo, Yamaguchi, or Miyazaki prefecture, despite the fact that the number of cases was small. There may have been an interruption in transmission, such as a loss of the host tick species, between the major and minor genotypes.

Taken together, the findings of the present phylogenetic analysis suggest novel geographic, evolutionary, and genotypic features of SFTSV, and the sequence results demonstrate that phylogenetic analyses of not only the patient serum samples but also the in vitro isolates can be used for experimental studies, because the isolates obtained in vitro retain their original characteristics.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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