Variable region of the 3′ UTR is a critical virulence factor in the Far-Eastern subtype of tick-borne encephalitis virus in a mouse model

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Tick-borne encephalitis virus (TBEV) is a major arbovirus that causes thousands of cases of severe neurological illness in humans annually. However, virulence factors and pathological mechanisms of TBEV remain largely unknown. To identify the virulence factors, we constructed chimeric viruses between two TBEV strains of the Far-Eastern subtype, Sofjin-HO (highly pathogenic) and Oshima 5-10 (low pathogenic). The replacement of the coding region for the structural and non-structural proteins from Sofjin into Oshima showed a partial increase of the viral pathogenicity in a mouse model. Oshima-based chimeric viruses with the variable region of the 3′ UTR of Sofjin, which had a deletion of 207 nt, killed 100% of mice and showed almost the same virulence as Sofjin. Replacement of the variable region of the 3′ UTR from Sofjin into Oshima did not increase viral multiplication in cultured cells and a mouse model at the early phase of viral entry into the brain. At the terminal phase of viral infection in mice, the virus titre of the Oshima-based chimeric virus with the variable region of the 3′ UTR of Sofjin reached a level identical to that of Sofjin and showed a similar histopathological change in the brain tissue. This is the first report to show that the variable region of the 3′ UTR is a critical virulence factor in mice. These findings encourage further study to understand the mechanisms of the pathogenicity of TBEV, and to develop preventative and therapeutic strategies for tick-borne encephalitis.

INTRODUCTION
Tick-borne encephalitis virus (TBEV; genus Flavivirus; family Flaviviridae) causes fatal encephalitis in humans. It is a major arbovirus that causes thousands of cases of severe neurological illness annually (Lindquist & Vapalahti, 2008; Mansfield et al., 2009). Tick-borne encephalitis is a significant public health problem in endemic areas of European and Asian countries (Bazan & Fletterick, 1989).

TBEV is a positive-stranded RNA virus with a genome of ~11 kb that encodes a long polyprotein in a single ORF, flanked by 5′ and 3′ UTRs. The corresponding polyprotein is processed into structural proteins, i.e. capsid (C), pre-membrane (prM) and envelope (E) proteins, as well as non-structural (NS) proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Heinz & Allison, 2003). The genome contains the 5′ and 3′ UTRs. The C protein is associated with the genome RNA packaging of TBEV (Kofler et al., 2002, 2003). The membrane (M) protein, which is translated initially as a precursor protein known as prM (Lobigs & Mullbacher, 1993), forms a heterodimer with the E protein, adding folding and maturation of the E protein. It is known that the E protein is responsible for binding to cellular receptors (Kopecky et al., 1999; Koziolovskaya et al., 2010; Navarro-Sanchez et al., 2003). The NS proteins play roles in genome replication and the processing of viral proteins. NS3 functions as a protease (Bazan & Fletterick, 1989; Fischl et al., 2008) and helicase (Matusan et al., 2001), and NS5 functions as a methyltransferase (Egloff et al., 2002) and RNA-dependent RNA polymerase (Park et al., 2007). The 5′ and 3′ UTRs are believed to be associated with viral genome replication (Khromykh et al., 2001; Kofler et al., 2006).

Based on phylogenetic analysis, TBEV can be divided into three subtypes: Far-Eastern, European and Siberian. Each subtype causes different symptoms and mortality (Gritsun et al., 2003). The Far-Eastern subtype is also known as Russian spring summer encephalitis virus and is prevalent in Far-Eastern Russia. This subtype causes severe neural disorders, such as encephalitis and meningoencephalitis.

Two supplementary tables are available with the online version of this paper.
Fig. 1. Effect of replacement of the TBEV region encoding most of the structural proteins on pathogenicity in mice. (a) Schematic representation of the genomes of recombinant Sofjin-IC and Oshima-IC viruses. Sofjin-IC/oshimaCME and Oshima-IC/sofjinCME were constructed by replacement of the coding region for most structural proteins (nt 240–2291) with that of Oshima-IC-pt and Sofjin-IC-pt, respectively. Sofjin-IC and Oshima-IC regions are shown in grey and white, respectively. (b) Growth curve of each virus in mouse neuroblastoma (NA) cells. NA cells were infected with each virus at an m.o.i. of 1. Viral titres at each time point were determined in baby hamster kidney (BHK-21) cells. The data are the means ± SD of three independent experiments. *At 24 h post-infection (p.i.), Sofjin-IC/oshimaCME and Oshima-IC/sofjinCME showed significant differences from Oshima-IC-pt and Sofjin-IC-pt (P < 0.05). †At 48 h p.i., a significant difference was observed between Sofjin-IC-pt and both chimeric viruses, and Oshima-IC/sofjinCME showed a significant difference from Oshima-IC-pt (P < 0.05). (c) Survival of mice inoculated with Sofjin-IC, Oshima-IC and the chimeric viruses. Mice were inoculated subcutaneously with 1000 p.f.u. of virus as indicated by the symbols.
with a higher mortality rate up to 30% (Bredenbeek et al., 2003; Ecker et al., 1999). The European subtype produces biphasic febrile illness and milder encephalitis, and the mortality rate is <2% (Dumpis et al., 1999). The Siberian subtype also causes less severe disease (case mortality rate 7–8%) than the Far-Eastern subtype and is often associated with chronic disease (Gritsun et al., 2003). The viral factors that determine the difference of the pathogenicities among the subtypes remain unknown.

The virus strain Sofjin-HO was isolated from a patient in Russia in 1937 and has been used as a prototype of the Far-Eastern subtype (Barkhash et al., 2010). It is also known to be highly pathogenic in a mouse model. The strain Oshima 5-10 was isolated from a sentinel dog in 1995 in the area in which a human case of tick-borne encephalitis was reported in Japan and was classified as the Far-Eastern subtype of TBEV. Oshima 5-10 is less virulent than Sofjin-HO in a mouse model (Bredenbeek et al., 2003; Chiba et al., 1999; Goto et al., 2002). The nucleotide homology between Oshima 5-10 and Sofjin-HO is high (96%) with differences of only 44 aa and a deletion of 207 nt in the 3’ UTR of Sofjin-HO (Tables S1 and S2, available in the online Supplementary Material). However, no information exists concerning the detailed mechanisms of different virulence in the two closely related strains, although they exhibit a high homology. Identifying the genetic factors associated with the different virulence is expected to facilitate elucidation of the mechanism of pathogenicity of TBEV.

Infectious cDNA clones are useful in investigating the genetic determinants of flavivirus replication and pathogenicity. Infectious cDNA clones have been generated for multiple flaviviruses, including yellow fever virus, West Nile virus, Dengue virus, Japanese encephalitis virus, Omsk haemorrhagic fever virus and TBEV (Bredenbeek et al., 2003; Mandl et al., 1997; Puri et al., 2000; Shi et al., 2002; Yoshii et al., 2011; Yun et al., 2003). In previous studies, we constructed full-length infectious cDNA clones of the Far-Eastern subtype Oshima 5-10 and Sofjin-HO strains (Hayasaka et al., 2004a, b; Takano et al., 2011).

In the present study, we constructed chimeric viruses between the infectious cDNA clones of the Far-Eastern subtype Sofjin-HO and Oshima 5-10 strains. The virulence of the chimeric viruses was investigated subsequently in a mouse model. We showed that the 3’ UTR is an important factor that determines the virulence of the Far-Eastern subtype of TBEV.

## RESULTS

### Replacement of the coding region for the structural proteins had no effect on virulence

The structural proteins of flaviviruses, especially the E proteins, have been reported to be important for virulence (Kopecký et al., 1999; Kozlovskaya et al., 2010; Navarro-Sanchez et al., 2003). To examine whether the structural proteins are determinants of virulence in mice, Sofjin-IC/oshimaCME and Oshima-IC/sofjinCME were constructed by replacement of the coding region for most of the structural proteins (nt 240–2291) with that of Oshima-IC-pt.

### Table 1. Morbidity and mortality of mice infected with Sofjin-IC-pt, Oshima-IC-pt or the recombinant viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Onset of disease (days)</th>
<th>Survival time (days)</th>
<th>Morbidity (%)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sofjin-IC-pt</td>
<td>7.7 ± 0.8†</td>
<td>9.0 ± 1.5†</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Oshima-IC-pt</td>
<td>11.9 ± 1.7</td>
<td>18.4 ± 5.3</td>
<td>90</td>
<td>50</td>
</tr>
<tr>
<td>Sofjin-IC/oshimaCME</td>
<td>8.8 ± 0.4†</td>
<td>8.4 ± 0.5†</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Oshima-IC/sofjinCME</td>
<td>9.6 ± 1.8</td>
<td>15.0 ± 6.2</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>Oshima-IC/sofjin 5’UTR-CN</td>
<td>8.7 ± 1.3†</td>
<td>13.5 ± 4.4</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Oshima-IC/sofjin NS1-2A N</td>
<td>9.3 ± 1.5†</td>
<td>13.7 ± 3.2</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>Oshima-IC/sofjin NS2A/G-4B N</td>
<td>8.4 ± 0.7†</td>
<td>11.0 ± 2.4†</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>Oshima/sofjin NS2A C</td>
<td>11.5 ± 2.6</td>
<td>16</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>Oshima/sofjin NS3 N</td>
<td>8.2 ± 1.1†</td>
<td>12.9 ± 4.7</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Oshima/sofjin NS3 N</td>
<td>9.8 ± 2.0</td>
<td>13.3 ± 1.5</td>
<td>90</td>
<td>30</td>
</tr>
<tr>
<td>Oshima/sofjin NS4 B N</td>
<td>9.5 ± 1.6</td>
<td>18.0 ± 5.7</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>Oshima-IC/sofjin NS4B C-5 N</td>
<td>9.3 ± 0.7†</td>
<td>13.9 ± 2.5</td>
<td>100</td>
<td>70</td>
</tr>
<tr>
<td>Oshima-IC/sofjin NS5-3’UTR</td>
<td>7.7 ± 0.7†</td>
<td>8.7 ± 1.2†</td>
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<td>100</td>
</tr>
<tr>
<td>Oshima-IC/NS5-778L</td>
<td>10.0 ± 0.8</td>
<td>17.8 ± 5.0</td>
<td>80</td>
<td>40</td>
</tr>
<tr>
<td>Oshima-IC/NS5-827S</td>
<td>8.9 ± 1.0†</td>
<td>14.9 ± 4.9</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>Oshima-IC/NS5-832A</td>
<td>9.6 ± 1.5*</td>
<td>15.5 ± 4.7</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>Oshima-IC/NS5-862K</td>
<td>9.8 ± 1.8*</td>
<td>17.4 ± 5.7</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>Oshima-IC/sofjin3’UTR_vari</td>
<td>8.6 ± 0.5†</td>
<td>10.4 ± 1.6*</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Oshima-IC/sofjin3’UTR_core</td>
<td>9.6 ± 1.8</td>
<td>13.7 ± 4.5</td>
<td>100</td>
<td>60</td>
</tr>
</tbody>
</table>

Five adult C57BL/6 mice were infected with Sofjin-IC/oshimaCME and Oshima-IC/sofjinCME, and 10 mice were infected with the other viruses. Significant differences between Oshima-IC and the other viruses: *P<0.05 or †P<0.01, respectively.

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Fig. 2. Effect of replacement of TBEV UTRs and the region encoding NS proteins on the pathogenicity in mice. (a) Schematic representation of the genomes of the chimeric viruses. Each Oshima-based virus was constructed by replacement of the 5′ UTR and the N terminus of C (5′UTR-CN), the C-terminal region of E and NS1 and the N-terminal region of NS2A (NS1-2AN), NS2AC-4BN, NS4BC-5N, NS5C-3′UTR).
the C terminus of NS2A and NS3 and the N terminus of NS4B (NS2A<sup>C</sup>–4B<sup>N</sup>), the C terminus of NS4B and the N terminus of NS5 (NS4B<sup>C</sup>–5<sup>N</sup>), or the C terminus of NS5 and the 3′ UTR (NS5<sup>C</sup>–3′UTR) with the respective regions of Sofjin-IC. The Sofjin-IC and Oshima-IC regions are shown in grey and white, respectively. (b) Growth curve of each virus in NA cells. NA cells were infected with each virus at an m.o.i. of 1. Viral titres at each time point were determined in BHK-21 cells. The data are the means ± SD of three independent experiments. *At 24 h p.i., Oshima-IC/sofjin NS1-2<sup>A</sup>, 5′UTR-C<sup>N</sup> and NS4B<sup>C</sup>–5<sup>N</sup> showed significant differences from Oshima-IC-pt, and Oshima-IC/sofjin NS5<sup>C</sup>–3′UTR and NS4B<sup>C</sup>–5<sup>N</sup> showed significant differences from Sofjin-IC-pt (P < 0.05). †At 48 h p.i., a significant difference was observed between Sofjin-IC-pt and Oshima-IC/sofjin 5′UTR-C<sup>N</sup> or NS5<sup>C</sup>–3′UTR, and between Oshima-IC-pt and the other viruses (P < 0.05). (c) Survival of mice inoculated with Sofjin-IC-pt, Oshima-IC-pt and the chimeric viruses. Mice were inoculated subcutaneously with 1000 p.f.u. of virus as indicated by the symbols.

and Sofjin-IC-pt, respectively (Fig. 1a). Although relatively lower growth was observed in the chimeric viruses, intact viruses were recovered (Fig. 1b).

The pathogenicity of the recombinant viruses was examined in a mouse model. C57BL/6 mice were infected subcutaneously with 1000 p.f.u. of Sofjin-IC-pt, Oshima-IC-pt, Sofjin-IC/oshimaCME or Oshima-IC/sofjinCME virus and survival was recorded for 28 days. The mice inoculated with each virus showed general signs of illness, such as reduced body weight, ruffled fur, and neurological signs of trembling and hind-limb paralysis; however, the survival time was longer and the mortality rate was lower in the mice infected with Oshima-IC-pt than in those infected with Sofjin-IC-pt (Fig. 1c, Table 1). The viruses in which the coding region for the structural protein were replaced (Sofjin-IC/oshimaCME or Oshima-IC/sofjinCME) showed virulence similar to that of the parental Sofjin-IC-pt or Oshima-IC-pt, regarding the survival curve, mean survival time and mortality in mice (Fig. 1c, Table 1). The results suggested that the difference in virulence between Sofjin and Oshima was not due to the structural proteins.

**C terminus of NS5 and the 3′ UTR are associated with the difference in virulence between Sofjin and Oshima**

As the replacement of the coding region for structural proteins did not affect the virulence, the other regions were investigated next. Recombinant Oshima-IC viruses were generated by partial replacement of the regions except the coding sequence for structural proteins, as shown in Fig. 2(a). The growth of each chimeric virus was higher than that of the parental Oshima strains (Fig. 2b). Mice were then infected with these recombinant viruses. Compared with the Oshima-IC-pt virus, mice infected with each recombinant virus showed a higher mortality and shorter survival time. However, only mice infected with the Oshima-IC/sofjin NS5<sup>C</sup>–3′UTR virus, in which the coding regions for the C terminus of NS5 and 3′ UTR were replaced with those of Sofjin-IC, showed a similar virulence to that of mice infected with Sofjin-IC-pt, regarding the survival curve, days of onset, mean survival time (significantly shorter than that of Oshima-IC-pt) and mortality (Fig. 2c, Table 1). These results suggested that the C terminus of NS5 and/or the 3′ UTR was important for the difference in virulence between the Sofjin-HO and Oshima 5-10 strains.

As Oshima-IC/sofjin NS2A<sup>C</sup>–4B<sup>N</sup> also showed a high pathogenicity with a short survival time, recombinant Oshima-IC viruses with replacement of the genes for NS2A, the N-terminal or C-terminal region of NS3, or the N terminus of NS4B were constructed (Fig. 3). No difference was noted in the amino acids of NS2B and NS4A between the Sofjin-HO and Oshima 5-10 strains. The growth of each chimeric virus was almost similar to that of the parental Oshima strain (Fig. 3b). The mortality of mice was 100% following infection of the chimeric virus with the N terminus of NS3. However, compared with Sofjin-IC-pt, the days to onset and survival time were longer in mice infected with the chimeric virus. The mice infected with the other viruses showed survival curves similar to the mice infected with Oshima-IC-pt, and no significant difference was found in the mean survival time between each virus and the parental Oshima-IC-pt. These results indicated that the difference in virulence between the Sofjin and Oshima strains could also be attributed to the N terminus of NS3, which encodes a serine protease.

**Partial deletion of the variable region of the 3′ UTR affects virulence**

There are 4 aa differences in the C terminus of NS5 and the nucleotide differences in the 3′ UTR. The 3′ UTR can be divided into two regions: the ‘variable region’, which varies among TBEV strains, and the ‘core element’, which is highly conserved in its sequence. In the variable region, there are 2 nt differences between the Sofjin and Oshima strains. A deletion of 207 nt is present in the variable region of Sofjin, as shown in Fig. 4(a). In the core element, 12 nt differences are evident between the two strains. To identify the factor(s) that affects the virulence in the coding regions for the C terminus of NS5 and the 3′ UTR, we constructed recombinant Oshima-IC viruses with a single amino-acid substitution in NS5 and replacement of the variable region or core element of the 3′ UTR, as described in Fig. 4(a). The virus titre of the supernatant of Sofjin-IC-pt-infected cells was significantly higher than that of each
Fig. 3. Effect of replacement of the TBEV region encoding NS proteins (NS2A, NS3 and NS4B) on pathogenicity in mice. (a) Schematic representation of the genomes of the chimeric viruses. Each Oshima-based virus was constructed by the NS2A, the N- and C-terminal region of NS3, and the N-terminal region of NS4B with those of Sofjin-IC. The Sofjin-IC and Oshima-IC
Effect of the deletion in the variable region of the virulence between the Sofjin and Oshima strains.

Suggested that the deletion in the variable region of the 3' UTR is an important determinant of the difference in virulence between the Sofjin and Oshima strains. No significant difference in each chimeric virus and Oshima-IC-pt viral titres was found (Fig. 4b). Mice were then infected with each recombinant virus. Only the Oshima-IC/sofjin3'UTR_vari virus, in which the variable region was replaced with that of Sofjin-IC-pt, killed 100% of mice and showed almost identical virulence to that of Sofjin-IC-pt virus, in terms of the survival curve, days of onset and mortality. Conversely, the other recombinant viruses showed a similar virulence to that of the mice infected with Oshima-IC-pt (Fig. 4c, Table 1). These results suggested that the deletion in the variable region of the 3' UTR is an important determinant of the difference in virulence between the Sofjin and Oshima strains.

**DISCUSSION**

In the present study, the important determinants of virulence were identified between the Far-Eastern subtype Sofjin and Oshima strains of TBEV. We showed that multiple viral factors affected the virulence cumulatively and that the variable region of the 3' UTR was a critical virulence determinant.

The E protein is thought to play a key role in determining the virulence of TBEV (Mandl, 2005). The E protein is expressed on the surface of mature virions and mediates virus entry into the host cell by binding to cell surface molecules (Heinz & Allison, 2003). The E protein has been suggested to be a crucial determinant of tissue tropism and neuropathogenesis during flavivirus infection. Amino acid changes in the E protein have been reported to affect the neurovirulence and neuroinvasiveness of tick-borne flaviviruses (Goto et al., 2003; Kozlovskaya et al., 2010; Mandl et al., 2001; Rumyantsev et al., 2006). However, the structural proteins, including the E protein, were not associated with the different virulence between the Sofjin-HO and Oshima 5-10 strains.

Replacement of the N terminus of NS3 increased virulence in mice. The flavivirus NS3 encodes a serine protease domain at its N terminal that is required for cleavage of the polyprotein during viral replication (Lescar et al., 2008). It combines with NS2B and forms the NS2B–NS3 protease complex as the activated serine protease (Bazan & Fletterick, 1989; Chambers et al., 1990; Gorbalenya et al., 1989). Several amino acid substitutions in the protease domain of NS3 can influence the activity of the enzyme and the virulence of TBEV (Chiba et al., 1999; Potapova et al., 2012; Rážek et al., 2008). Seven amino acid differences in the N terminus of NS3 exist between Sofjin-HO and Oshima 5-10. A previous report suggested that the serine—phenylalanine substitution at position 45 affects TBEV pathogenicity (Chiba et al., 1999). An identical substitution was also
Fig. 4. Effect of substitutions of TBEV amino acids in NS5 and replacement of the 3′ UTR. (a) Schematic representation of the genome of recombinant viruses. Single amino-acid substitutions were introduced at NS5 positions 778 (NS5-778L), 827 (NS5-827S), 832 (NS5-832A) and 862 (NS5-862K) of Oshima-IC. The grey and white arrowheads indicate amino acids derived from Sofjin-IC-pt and Oshima-IC-pt, respectively. Oshima-IC/sofjin3′ UTR_vari and Oshima-IC/sofjin3′ UTR_core are
Oshima-IC chimeric viruses in which the variable region and core element of the 3′ UTR were replaced with those of Sofjin-IC. The grey lines indicate the regions derived from the 3′ UTR of Sofjin-IC. The broken line indicates the region lacking in Sofjin-IC-pt. (b) Growth curve of each virus in NA cells. NA cells were infected with each virus at an m.o.i. of 1. Viral titres at each time point were determined in BHK-21 cells. The data are the means ± SD of three independent experiments. *At 24 h p.i., the chimeric viruses except for Oshima-IC/sofjin3′UTR_core showed significant differences from Sofjin-IC-pt (P<0.05). † At 48 h p.i., significant differences were observed between Sofjin-IC-pt and the other viruses (P<0.01). No significant difference between Oshima-IC-pt and each chimeric virus was observed at 24 and 48 h p.i. (c) Survival of mice inoculated with Sofjin-IC-pt, Oshima-IC-pt and the chimeric viruses. Mice were inoculated subcutaneously with 1000 p.f.u. of virus as indicated by the symbols.

observed between Sofjin-HO and Oshima 5-10 strains. Therefore, this substitution might be associated with partially affecting the difference in virulence between Sofjin-HO and Oshima 5-10 we report here.

Replacement of the variable region of the 3′ UTR of Oshima with that of Sofjin resulted in a marked increase in virulence. The 3′ UTR of TBEV consists of two distinct domains: the 5′-terminal variable region and 3′-terminal core element (Gritsun et al., 1997; Wallner et al., 1995). The core element shows a high degree of sequence conservation among TBEV strains and contains sequences necessary for viral genome replication, such as cyclization sequence (Kofler et al., 2006). The sequence of the variable region varies among the TBEV strains and the role of this region is unclear. In a study of European TBEV subtype strains, deletion of the entire 3′ UTR variable region did not affect viral multiplication in cultured cells or virulence in mice (Mandl et al., 1998). The discrepant results obtained in the present study might be due to the use of different strains. As the Neudoerfl strain used in the study of Mandl et al. (1998) was highly virulent in the mouse model (LD_{50}≤10), it is possible that deletion of the whole variable region did not result in an increase in virulence. Additionally, the Neudoerfl strain contains an insertion of a poly(A) sequence in the variable region (Mandl et al., 1998) that is not present in most other TBEV strains. It is also possible that the addition of the poly(A) sequence

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**Fig. 5.** Effects of the replacement of the variable region on viral multiplication in organs. Mice were infected with 1000 p.f.u. of Sofjin-IC-pt, Oshima/sofjin3′UTR_vari and Oshima-IC-pt as indicated by the symbols. Virus titres in the (a) blood, (b) spleen, and (c) brain at the indicated days after infection were determined by plaque assays. The horizontal dashed lines indicate the limits of detection for the assay (100 p.f.u. ml⁻¹). Error bars represent the SD (n=3). An asterisk (*) or dagger (†) denotes a significant difference compared with Oshima-IC-pt or Sofjin-IC-pt, respectively (P<0.05).
might affect the function of the 3' UTR, as observed for the partial deletion of the 3' UTR in Sofjin, resulting in increased virulence. Nevertheless, the deletion of the variable region in the 3' UTR of the Far-Eastern subtype of TBEV resulted in increased virulence in mice. This result suggested an unidentified role of the variable region in the viral pathogenicity.

Replacement of the variable region of the 3' UTR did not affect viral replication in cell culture. It also did not increase viral multiplication in the mouse brain by 7 days p.i. However, by 9 days p.i., the viral titre of the chimeric virus with the variable region of the Sofjin strain increased markedly to a level identical to that of the Sofjin strain as evidenced by severe pathological changes in the brain. These data suggested involvement of the variable region in regulation of the host response, which in turn affected the viral replication in the brain. A recent study of West Nile virus and Japanese encephalitis virus reported that the subgenomic flavivirus RNA (sfRNA) was mediated from the 3' UTR as a product of the genomic RNA degradation by host exoribonuclease and that sfRNA mediated pathogenicity by interfering with host protective responses, such as the RNA interference machinery and type I IFN response (Pijlman et al., 2008; Schnettler et al., 2012). Therefore, the deletion in the 3' UTR of Sofjin-HO may affect the function, amount or stability of sfRNA.

The sequence of the 3’ UTR variable region varies among TBEV strains; however, the role of this region remains unknown. Strains freshly isolated from ticks and wild rodents do not have a deletion in the variable region, and this region is considered to be essential for the natural transmission cycle of TBEV (Bredenbeek et al., 2003). Conversely, deletions in the variable region of 3’ UTR were found in many Far-Eastern subtype isolates from human patients (Leonova et al., 2013). Mandl et al. (1998) reported that the deletion in the 3’ UTR occurred during passage in mammalian cell culture or in mice. Together, these reports suggest that the deletion caused by adaptation or selection in mammalian cells affects replication, resulting in an increased virulence in mammals.

In conclusion, we report here that the different virulence between Sofjin and Oshima is determined by multiple viral factors cumulatively, and the variable region of the 3’ UTR is an important determinant of pathogenicity in mice. Deletion in the region affected multiplication in the brain, resulting in the severe pathological changes associated with the Far-Eastern subtype TBEV. These findings encourage further research to identify the pathogenic mechanisms of TBEV, and to develop prevention and therapeutic strategies for TBE, such as the development of an attenuated live vaccine and design of targets of antiviral drugs.

**METHODS**

**Cells.** Baby hamster kidney (BHK-21) cells and mouse neuroblastoma (NA) cells were grown in Eagle’s minimal essential medium (MEM), supplemented with 8 and 10 % FCS, respectively.

**Viruses.** Viruses were prepared from infectious cDNA clones. Infectious cDNA plasmids of parental Sofjin-IC and Oshima-IC (Sofjin-IC-pt and Oshima-IC-pt), which encode the full-length cDNA of the TBEV Sofjin-HO (GenBank accession no. AB062064) and Oshima 5-10 (GenBank accession no. AB062063) strains, respectively, were prepared as described previously (Hayasaka et al., 2004a, b; Takano et al., 2011).

Infectious cDNA plasmids of the recombinant viruses listed in Figs 1 and 2 were constructed by the replacement of indicated regions between Sofjin-IC-pt and Oshima-IC-pt using the indicated restriction enzyme sites. To construct infectious cDNA plasmids of the recombinant viruses listed in Fig. 3(a), the DNA fragment with the indicated nucleotides of Sofjin was amplified by fused PCR and was inserted into Oshima-IC using the AgeI and AatII restriction enzyme sites. Oshima-IC/sofjinNS2A<sup>C</sup> was constructed by site-directed mutagenesis as described below. To construct infectious cDNA plasmids of recombinant Oshima-IC viruses with substitutions of single amino acids, site-directed mutations were introduced into position 225 of NS2A, and positions 778, 827, 832 and 862 of NS5 using the Quick Change II XL site-directed mutagenesis kit (Agilent Technologies) as shown in Figs 3(a) and 4(a).

To construct infectious cDNA plasmids of the recombinant virus Oshima-IC/sofjin3'UTR_vari and Oshima-IC/sofjin3'UTR_core, the fragment (nt 9830–1100 of Oshima-IC) with the variable region (nt 10377–10551) and the core element (nt 10352–10894) of Sofjin-IC.
were amplified by fused PCR, and inserted into Oshima-IC using Ascl and SpeI, as shown in Fig. 4(a). The differences of nucleotide and amino acids between Sofjin, Oshima and each recombinant virus are shown in Tables S1 and S2.

The infectious cDNA plasmids were linearized with SpeI and transcribed into RNA using the mMESSAGE mACHINE SP6 Kit (Life Technology) as described previously (Gritsun & Gould, 1995). The mRNA samples were treated with DNase I and precipitated with LiCl. The precipitated RNA was dissolved in 30 μl DEPC-treated water. BHK-21 cells were transfectd with mRNA using a TransIT-mRNA Transfection Kit (Mirus Bio) as described previously (Hayasaka et al., 2004a). Two days post-transfection, recombinant viruses in the supernatant of the RNA-transfected cells were harvested and stored at –80 °C.

Virus titration. Plaque assays were carried out with BHK-21 cells using 12-well plates. The cells were inoculated with serial 10-fold dilutions of organ suspensions or culture medium from infected cells (100 μl), and they were incubated for 1 h at 37°C before 1.5% carboxy methyl cellulose in MEM (1 ml well–1) was added. Incubation was continued for 3–4 days and the monolayers were stained with 0.1% crystal violet solution. Plaques were counted and infectivity titres were expressed as p.f.u. ml–1.

Growth curve in cell culture. Subconfluent NA cells were grown in 24-well plates. Cells were inoculated with each virus at an m.o.i. of 1. Cells were incubated at 37 °C in 5% CO2. The supernatant was harvested at 24 and 48 h post-inoculation and stored in aliquots at –80 °C.

Animal model. Five-week-old female C57BL6 mice (Jackson ImmunoResearch) were inoculated subcutaneously with 1000 p.f.u. of virus. Morbidity was defined as the appearance of 10% weight loss. Surviving mice were monitored for 28 days p.i. to obtain survival curves and mortality rates. For the analysis of viral distribution in tissues, serum, brain and spleen were collected from the mice on days 1, 3, 5, 7 and 9 p.i. Organs were individually weighed and homogenized, and prepared as 10% suspensions (w/v) in PBS with 10% FCS. The suspensions were clarified by centrifugation (4000 r.p.m. for 5 min, 4 °C) and the supernatants were titrated by plaque assay on BHK-21 cells. All procedures were performed according to the guidelines of the Animal Care and Use Committee of Hokkaido University.

Histopathological examination. Three mice infected with 103 p.f.u. of TBEV were killed at 7 and 9 days p.i., and formalin-fixed brains were routinely processed and embedded in paraffin, sectioned, and stained with haematoxylin and eosin as described previously (Sunden et al., 2010). Immunohistochemical detection of TBEV antigens was performed using rabbit polyclonal antibodies against E protein to detect TBEV antigens (Yoshii et al., 2004).

Statistical analysis. P values of differences in virus titres were calculated using an unpaired Student’s t-tests.

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