The variable region of the 3' untranslated region is a critical virulence factor in the Far-Eastern subtype of tick-borne encephalitis virus in mouse model

Running title: Role of variable region as a virulence factor in TBEV

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Summary

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' untranslated region (3'-UTR) of Sofjin, which had a deletion of 207 nucleotides, killed 100% of mice and showed almost same virulence with Sofjin. Replacement of the variable region of 3'-UTR from Sofjin into Oshima did not increase viral multiplication in cultured cell 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 titer 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 develop preventative and therapeutic strategies for TBE.

Introduction

Tick-borne encephalitis (TBE) virus, which is a member of the genus *Flavivirus* in the family *Flaviviridae*, causes fatal encephalitis in humans. It is a major arbovirus that causes thousands of cases of severe neurological illness annually. TBE is a significant public health problem in endemic areas of European and Asian countries (Bazan & Fletterick, 1989).

TBE virus (TBEV) is a positive-stranded RNA virus with a genome of ~11 kb that encodes a long polyprotein in a single open reading frame (ORF), flanked by 5' and 3'-untranslated regions (UTRs). The corresponding polyprotein is processed into structural proteins, i.e., capsid (C), pre-Membrane (prM), envelope (E) protein, as well as non-structural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Heinz & Allison, 2003). The genome contains the 5' and 3'-UTR. The C protein is associated with the genome RNA packaging of TBEV (Kofler *et al.*, 2002; Kofler *et al.*, 2003). The M protein, which is initially translated as a precursor protein known as prM (Lobigs & Mullbacher, 1993), forms a heterodimer with E protein, adding folding and maturation of the E protein. It is known that E protein is responsible for binding to cellular receptors (Kopecky *et al.*, 1999; Kozlovskaya *et al.*, 2010; Navarro-Sanchez *et al.*, 2003). The non-structural 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), RNA-dependent RNA polymerase (Park *et al.*, 2007). The 5' and 3'-UTR are believed to be associated with the 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 subtype. 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

66 meningoencephalitis with a higher mortality rate up to 30 % (Bredenbeek *et al.*, 2003; Ecker *et al.*,
67 1999). The European subtype produces biphasic febrile illness and milder encephalitis, and the
68 mortality rate is lower than 2% (Dumpis *et al.*, 1999). The Siberian subtype also causes less severe
69 disease (case mortality rate, 7 to 8%) than the Far-Eastern subtype and is often associated with
70 chronic disease (Gritsun *et al.*, 2003). It remains unknown about viral factors to determine the
71 difference of the pathogenicities among the subtypes.
72 The virus strain Sofjin-HO was isolated from a patient in Russia in 1937 and has been used as a
73 prototype of the Far-Eastern subtype (Barkhash *et al.*, 2010). It is also known to be highly
74 pathogenic in a mouse model. The strain Oshima 5-10 was isolated from a sentinel dog in 1995 in
75 the area in which a human case of TBE was reported in Japan, and was classified as the Far-Eastern
76 subtype of TBEV. Oshima 5-10 is less virulent than Sofjin-HO in a mouse model (Bredenbeek *et al.*,
77 2003; Chiba *et al.*, 1999; Goto *et al.*, 2002). The nucleotide homology between Oshima 5-10 and
78 Sofjin-HO is high (96%) with differences of only 44 amino acids and a deletion of 207 nucleotides
79 in the 3'-UTR of Sofjin-HO (Supplementary table 1 and 2). However, no information exists
80 concerning the detailed mechanisms of different virulence in the two closely related strains although
81 they exhibit a high homology. Identifying the genetic factors associated with the different virulence
82 is expected to facilitate elucidation of the mechanism of pathogenicity of TBEV.
83 Infectious cDNA clones are useful in investigating the genetic determinants of flavivirus
84 replication and pathogenicity. Infectious cDNA clones have been generated for multiple flaviviruses,
85 including yellow fever virus, West Nile virus, Dengue virus, Japanese encephalitis virus, Omsk
86 hemorrhagic fever virus and TBEV (Bredenbeek *et al.*, 2003; Mandl *et al.*, 1997; Puri *et al.*, 2000;
87 Shi *et al.*, 2002; Yoshii *et al.*, 2011; Yun *et al.*, 2003). In previous studies, we constructed full-length
88 infectious cDNA clones of the Far-Eastern subtype Oshima 5-10 and Sofjin-HO strains (Hayasaka *et*
89 *al.*, 2004a; Hayasaka *et al.*, 2004b; 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 subsequently investigated 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 (Kopecky *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 (nucleotides 240-2291) with that of Oshima-IC-pt and Sofjin-IC-pt, respectively (Fig.1(a)). Although relatively lower growth was observed in the chimeric viruses, intact viruses were recovered (Fig.1(b)).

The pathogenicity of the recombinant viruses was examined in a mouse model. C57BL/6 mice were infected subcutaneously with 1,000 pfu 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 neurologic 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.1(b) and 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 parental Sofjin-IC-pt or Oshima-IC-pt, regarding the survival curve, average of survival time and mortality in mice (Fig.1(b) and Table 1). The results suggested that the difference in virulence

between Sofjin and Oshima was not due to the structural proteins.

The C-terminus of NS5 and the 3'-UTR are associated with the difference in virulence between Sofjin and Oshima

Because the replacement of the coding region for structural proteins did not affect the virulence, the other regions were next investigated. 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.2(b)). 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/NS5^{C}-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, average survival time (significantly shorter than that of Oshima-IC-pt) and mortality (Fig.2(b) and Table 1). These results suggested that the C-terminus of NS5 and/or 3'-UTR was important for the difference in virulence between the Sofjin-HO and Oshima 5-10 strains.

Because Oshima-IC/sofjinNS2A^{C}-4B^{N} 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.3(b)). 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

138 similar to the mice infected with Oshima-IC-pt, and no significant difference was found in the
139 average survival time between each virus and the parental Oshima-IC-pt. These results indicated that
140 the difference in virulence between the Sofjin and Oshima strains could also be attributed to the
141 N-terminus of NS3, which encodes a serine protease.
142
143 ***Partial deletion of the variable region of the 3'-UTR affects virulence***
144 There are four amino acid differences in the C-terminus of NS5 and the nucleotide differences in the
145 3'-UTR. The 3'-UTR can be divided into two regions: the “variable region” which varies among
146 TBEV strains, and the “core element” which is highly conserved in its sequence. In the variable
147 region, there are two nucleotide differences between the Sofjin and Oshima strains. A deletion of
148 207 nucleotides is present in the variable region of Sofjin, as shown in Fig.4 (a). The virus titer of
149 the supernatant of Sofjin-IC-pt infected cells was significantly higher than that of each chimeric
150 virus or Oshima-IC-pt infected cells. No significant difference in each chimeric virus and
151 Oshima-IC-pt viral titers was found (Fig.4(b)). In the core element, 12 nucleotide differences are
152 evident between the two strains. To identify the factor (s) that affects the virulence in the coding
153 regions for the C-terminus of NS5 and 3'-UTR, we constructed recombinant Oshima-IC viruses with
154 single amino acid substitution in NS5, and replacement of the variable region or core element of the
155 3'-UTR, as described in Fig.4 (a). Mice were then infected with each recombinant virus. Only the
156 Oshima-IC/3'-UTR_vari virus, in which the variable region was replaced with that of Sofjin-IC-pt,
157 killed 100% of mice and showed almost identical virulence to that of Sofjin-IC-pt virus, in terms of
158 the survival curve, days of onset and mortality. Conversely, the other recombinant viruses showed a
159 similar virulence to that of the mice infected with Oshima-IC-pt (Fig. 4(b) and Table 1). These
160 results suggested that the deletion in the variable region of the 3'-UTR is an important determinant of
161 the difference in virulence between the Sofjin and Oshima strains.

Effect of the deletion in the variable region of the 3'-UTR on viral multiplication and pathogenicity

We investigated the effect of the deletion in the variable region on viral multiplication in mouse neuroblastoma (NA) cells (Fig.5). The virus titer of the supernatant of Sofjin-IC-pt infected cells was significantly higher than that of Oshima-IC/Sofjin 3'-UTR_vari or Oshima-IC-pt infected cells. No significant difference in the Oshima-IC/Sofjin 3'-UTR_vari and Oshima-IC-pt viral titers was found. This result suggested that the deletion in the variable region did not affect viral multiplication in cultured cells. To examine the correlation between disease development and viral replication in organs, the viral loads in the blood, spleen, and brain were compared in mice inoculated with the Sofjin-IC-pt, Oshima-IC/3'-UTR_vari or Oshima-IC-pt viruses (Fig.6). Transient viremia was observed in the mice infected with each virus, which almost disappeared by 5 to 7 days post infection. Increases in viral replication were observed in the spleen after viremia (from 3 days post infection).

The virus was detected in the brain by 7 days post infection. The titer was reached 1.4×10^{7} pfu ml^{-1} at 7 days post infection in the mice inoculated with Sofjin-IC-pt, and was significantly higher than that in the mice infected with the Oshima-IC/Sofjin 3'-UTR_vari or Oshima-IC-pt virus ($P<0.05$). No significant difference between in titer in brains infected with Oshima-IC/Sofjin3'-UTR_vari and Oshima-IC-pt was found. However, at 9 days post infection, the virus titer in the brain infected with Oshima-IC/Sofjin3'-UTR_vari reached 8.3×10^{7} pfu ml^{-1}, a level almost identical to that in the brain infected with Sofjin-IC-pt, and significantly higher than that in the mouse brain infected with Oshima-IC-pt.

Histopathological features in mice infected with Oshima-IC-pt, Sofjin-IC-pt or Oshima/3'-UTR_vari at 7 and 9 days post infection were investigated (Fig.7). At day 7, there were

few pathological changes in mice infected with each virus (data not shown). However, at day 9, abundant viral antigens and pathological changes, such as inflammatory infiltrations, small hemorrhages, and necrotic or degenerated neurons were observed throughout the brains of mice infected with Sofjin-IC-pt and Oshima/3'-UTR_vari. Compared with Sofjin-IC-pt and Oshima/3'-UTR_vari infected mice brains, there were fewer virus-antigen-positive cells and mild pathological changes in the brains of mice infected with Oshima-IC-pt.

Taken together, these data suggested that the deletion in the variable region of the 3'-UTR enhanced virus multiplication and pathogenicity in the mouse brain.

Discussion

In the present study, the important determinants of virulence were identified between the Far-Eastern subtype strains of TBEV Sofjin and Oshima. 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). 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; Ruzek *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 to phenylalanine substitution at amino acid position 45 affects TBEV pathogenicity (Chiba *et al.*, 1999). An identical substitution was also 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 variable region varies among the strains of 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 the 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 use of different strains. Because the Neudoerfl strain used in the study of Mandl *et al* (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 of other TBEV strains. It is also possible that the addition of the poly-A sequence might affect the function of the 3'-UTR, as observed for the partial deletion of the 3'-UTR in Sofjin, resulting in increased virulence.

234 Nevertheless, the deletion of the variable region in the 3'-UTR of the Far-Eastern subtype of TBEV
235 resulted in an increased virulence in mice. This result suggested an unidentified role of the variable
236 region in the viral pathogenicity.

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

249 The sequence of the 3'-UTR variable region varies among TBEV strains; however, the role of
250 this region remains unknown. Strains freshly-isolated from ticks and wild rodents do not have a
251 deletion in the variable region, and this region is considered to be essential for the natural
252 transmission cycle of TBEV (Bredenbeek *et al.*, 2003). Conversely, deletions in the variable region
253 of 3'-UTR were found in many Far-Eastern subtype isolates from human patients (Leonova *et al.*,
254 2013). Mandl *et al* reported that the deletion in the 3'-UTR occurred during passage in mammalian
255 cell culture or in mice (Mandl *et al.*, 1998). Together, these reports suggest that the deletion caused
256 by adaptation or selection in mammalian cells affected replication, resulting in an increased
257 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 develop prevention and therapeutic strategies for TBE, such as development of an attenuated live vaccine and design of targets of anti- viral 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% fetal calf serum (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 (accession no. AB062064) and Oshima 5-10 (accession no. AB062063) strains, respectively, were prepared as described previously (Hayasaka *et al.*, 2004a; Hayasaka *et al.*, 2004b; Takano *et al.*, 2011).

Infectious cDNA plasmids of the recombinant viruses listed in Figs.1 and 2 were constructed by the replacement of the indicated regions between Sofjin-IC-pt and Oshima-IC-pt using the indicated restriction enzyme sites. To construct infectious cDNA plasmid of the recombinant viruses listed in Fig. 3(a), the DNA fragment with the indicated nucleotides of Sofjin was amplified by fused-polymerase chain reaction (PCR) and was inserted into Oshima-IC using the *Age*I and *Aat*II restriction enzyme sites. Oshima-IC/sofjinNS2A^{C} was constructed by site-directed mutagenesis as

282 described below. To construct infectious cDNA plasmids of recombinant Oshima-IC viruses with
283 substitutions of single amino acids, the site-directed mutations were introduced into the amino acid
284 position 225 of NS2A, and the amino acid positions 778, 827, 832 and 862 of NS5 using the Quick
285 Change II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, U.S.) as shown
286 in Figs. 3(a) and 4(a).
287 To construct infectious cDNA plasmids of the recombinant virus Oshima-IC/sofjin3'-UTR-vari
288 and Oshima-IC/sofjin3'-UTR-core, the fragment (nucleotides 9830-11100 of Oshima-IC) with the
289 variable region (nucleotides 10377-10551) and the core element (nucleotides 10552-10894) of
290 Sofjin-IC were amplified by fused PCR, and inserted into Oshima-IC using *Asc*I and *Spe*I, as shown
291 in Fig.4(a). The differences of nucleotide and amino acids between Sofjin, Oshima and each
292 recombinant virus were shown in Supplementary table 1 and 2.
293 The infectious cDNA plasmids were linearized with the *Spe*I and transcribed into RNA using the
294 mMESSAGE mMACHINE SP6 Kit (Life Technology, Carlsbad, CA, USA), as described previously
295 (Gritsun & Gould, 1995). The mRNA samples were treated with DNase I and precipitated with LiCl.
296 The precipitated RNA was dissolved in 30 µl of diethylpyrocarbonate-treated water. BHK-21 cells
297 were transfected with mRNA using a trans IT-mRNA Transfection Kit (Mirus Bio LLC, Madison,
298 WI, USA) as described previously (Hayasaka *et al.*, 2004a). Two days post transfection, recombinant
299 viruses in the supernatant of the RNA-transfected cells was harvested and stored at -80 °C.
300
301 **Virus titration.** Plaque assays were carried out with BHK-21 cells using 12-well plates. The cells
302 were inoculated with the serial 10-fold dilutions of organ suspensions or culture medium from
303 infected cells (100 µl), and they were incubated for 1 h at 37°C before 1.5% carboxy methyl
304 cellulose in MEM (1 ml well^{-1}) was added. Incubation was continued for 3-4 days, and the
305 monolayers were stained with 0.1% crystal violet in 10% formalin neutral buffer solution. Plaques

were counted, and infectivity titers were expressed as plaque-forming units (pfu) ml^{-1}.

Growth curve in cell culture. Subconfluent NA cells were grown in 24-well plates. Cells were inoculated with each virus at a multiplicity of infection (MOI) of 1. Cells were incubated at 37°C in 5% CO_2. 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 Immuno Research, West Grove, PA, USA) were inoculated subcutaneously with 1,000 pfu of viruses. Morbidity was defined as the appearance of 10% weight loss. Surviving mice were monitored for 28 days post infection 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 post infection. Organs were individually weighed and homogenized, and prepared as 10% suspensions (w/v) in phosphate-buffered saline with 10% FCS. The suspensions were clarified by centrifugation (4,000 rpm 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 the Hokkaido University.

Histpathological examination. Three mice infected with 10^3 pfu of TBEV were killed at 7 and 9 days post infection, 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 titers were calculated using an unpaired Student's t-tests.

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

	Onset of disease (days)	Survival time (days)	Morbidity (%)	Mortality (%)
Sofjin-IC-pt	7.7±0.8**	9.0±1.5**	100	100
Oshima-IC-pt	11.9±1.7	18.4±5.3	90	50
Sofjin-IC/oshimaCME	8.8±0.4**	8.4±0.5**	100	100
Oshima-IC/sofjinCME	9.6±1.8	15.0±6.2	100	60
Oshima-IC/sofjin5'UTR-C^N	8.7±1.3**	13.5±4.4	100	100
Oshima-IC/NS1-$2A^N$	9.3±1.5**	13.7±3.2	100	90
Oshima-IC/$NS2A^C$-$4B^N$	8.4±0.7**	11.0±2.4**	100	90
Oshima/sofjinNS$2A^C$	11.5±2.6	16	80	20
Oshima/sofjinNS3^N	8.2±1.1**	12.9±4.7	100	100
Oshima/sofjinNS3^C	9.8±2.0	13.3±1.5	90	30
Oshima/sofjinNS$4B^N$	9.5±1.6	18.0±5.7	100	80
Oshima-IC/$NS4B^C$-5^N	9.3±0.7**	13.9±2.5	100	70
Oshima-IC/$NS5^C$-3'UTR	7.7±0.7**	8.7±1.2**	100	100
Oshima-IC/NS5-778L	10.0±0.8	17.8±5.0	80	40
Oshima-IC/NS5-827S	8.9±1.0**	14.9±4.9	100	80
Oshima-IC/NS5-832A	9.6±1.5*	15.5±4.7	100	60
Oshima-IC/NS5862K	9.8±1.8*	17.4±5.7	100	50
Oshima-IC/3'-UTR-variable	8.6±0.5**	10.4±1.6*	100	100
Oshima-IC/3'-UTR-core	9.6±1.8	13.7±4.5	100	60

† Five adult mice C57BL/6 were infected with Sofjin-IC/oshimaCME and Oshima-IC/sofjinCME, and ten mice were infected with the others.

* or * * denotes a significant difference between Oshima-IC and the other viruses ($P<0.05$ or 0.01) , respectively.

Figure Legends

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 (nucleotides 240-2291) with that of Oshima-IC-pt and Sofjin-IC-pt, respectively. Sofjin-IC and Oshima-IC regions are shown in gray and white, respectively. **(b)** Growth curve of each virus in NA cells. NA cells were infected with each virus at multiplication of infection (MOI) of 1. Viral titers at each time point were determined in BHK-21 cells. The data is the average of three independent experiments. * At 24h post-infection, Sofjin-IC/oshimaCME and Oshima-IC/sofjinCME showed significant differences from Oshima-IC-pt and Sofjin-IC-pt ($P<0.05$). † At 48h post-infection, a significant difference was observed between Sofjin-IC-pt and both chimeric viruses, and Oshima-IC/sofjinCME showed 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 1,000 pfu of Sofjin-IC-pt (closed square), Sofjin-IC/oshimaCME (open square), Oshima-IC/sofjinCME (open circle) and Oshima-IC-pt (closed circle).

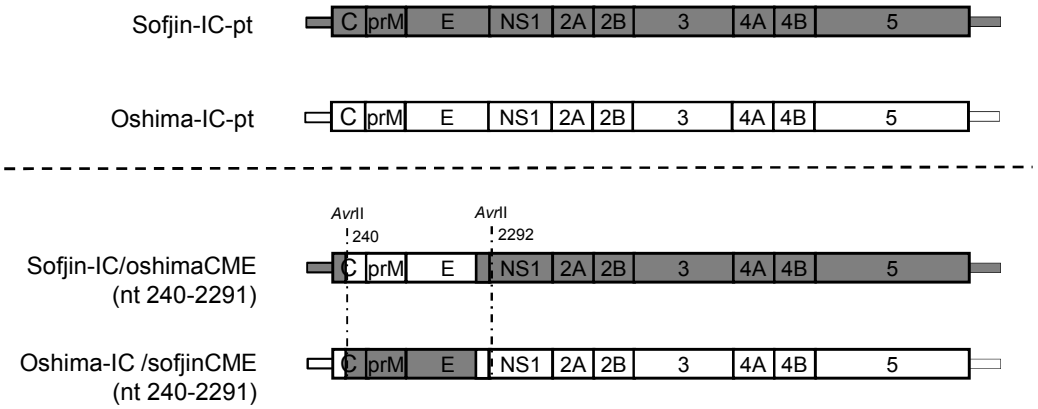
Fig.2 Effect of replacement of TBEV untranslated regions and the region encoding non-structural proteins on the pathogenicity in mice. (a) Schematic representation of the genomes of the chimeric viruses. Each Oshima-based virus was constructed by replacement of the 5'-UTR and the N-terminus of C (5'UTR-C^N), the C-terminal region of E and NS1 and the N-terminal region of NS2A (NS1-$2A^N$), the C-terminus of NS2A and NS3 and the N-terminus of NS4B ($NS2A^C$-$4B^N$), the C-terminus of NS4B and the N-terminus of NS5 ($NS4B^C$-5^N), or the C-terminus of NS5 and the 3'-UTR ($NS5^C$-3'UTR) with the respective region regions of Sofjin-IC. The Sofjin-IC- and

527 Oshima-IC regions are shown in gray and white, respectively**. (b)** Growth curve of each virus in NA
528 cells. NA cells were infected with each virus at multiplication of MOI of 1. Viral titers at each time
529 point were determined in BHK-21 cells. The data is the average of three independent experiments. *
530 At 24h post-infection, Oshima-IC/sofjin NS1-2A^{N}, 5'UTR-C^{N} and NS4B^{C}-5^{N} showed significant
531 difference from Oshima-IC-pt, and Oshima-IC/sofjin NS5^{C}-3'UTR, NS4B^{C}-5^{N} showed it from
532 Sofjin-IC-pt ($P<0.05$). † At 48h post-infection, a significant difference was observed between
533 Sofjin-IC-pt and Oshima-IC/sofjin 5'UTR-C^{N} or NS5^{C}-3'UTR, and between Oshima-IC-pt and the
534 other viruses ($P<0.05$). **(c)** Survival of mice inoculated with Sofjin-IC-pt, Oshima-IC-pt, and the
535 chimeric viruses. Mice were inoculated subcutaneously with 1,000 pfu of Sofjin-IC-pt (closed
536 square), Oshima-IC/sofjin5'UTR-C^{N} (closed triangle), Oshima-IC/sofjinNS1-2A^{N} (open triangle),
537 Oshima-IC/sofjinNS2A^{C}-4B^{N} (closed diamond), Oshima-IC/sofjinNS4B^{C}-5^{N} (open diamond),
538 Oshima-IC/sofjin NS5^{C}-3'UTR (open square), or Oshima-IC-pt (closed circle).
539
540
541 **Fig.3** Effect of replacement of the TBEV region encoding non-structural proteins (NS2A, NS3 and
542 NS4B) on the pathogenicity in mice. (a) Schematic representation of the genomes of the chimeric
543 viruses. Each Oshima-based virus was constructed by replacement of the NS2A, the N- and
544 C-terminal region of NS3 and the C-terminal region of NS4B with each of Sofjin-IC. The Sofjin-IC
545 and Oshima-IC regions are shown in gray and white, respectively**. (b)** Growth curve of each virus in
546 NA cells. NA cells were infected with each virus at multiplication of MOI of 1. Viral titers at each
547 time point were determined in BHK-21 cells. The data is the average of three independent
548 experiments. * At 24h post-infection, Oshima-IC/sofjin NS3^{C} and NS4B^{N} showed a significant
549 difference from Oshima-IC-pt, and Oshima-IC/sofjin NS2A^{C,} NS3^{N}, NS4B^{N} showed it from
550 Sofjin-IC-pt ($P<0.05$). † At 48h post-infection, a significant difference was observed between

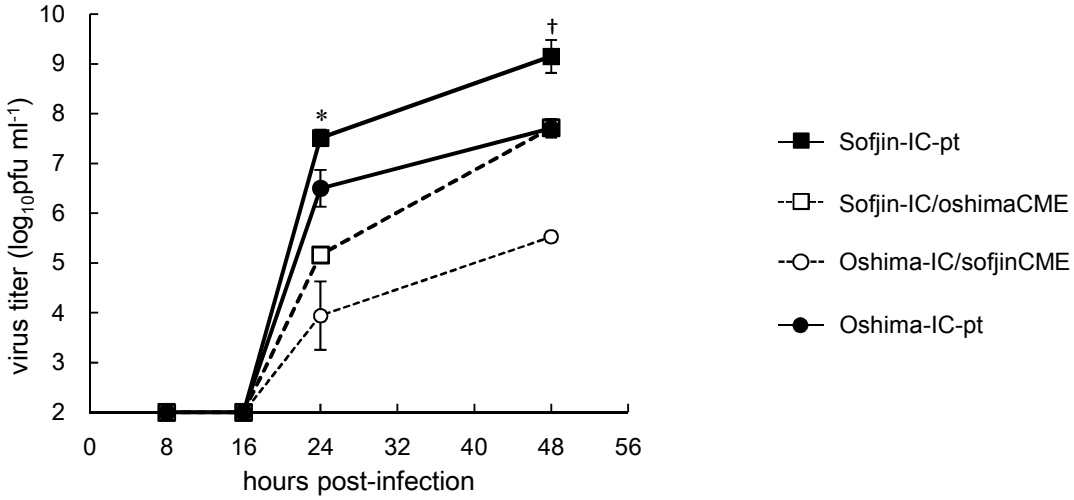
551 Sofjin-IC-pt and the other viruses (P<0.05). No significant differences between Oshima-IC-pt and
552 each chimeric virus was observed. **(c)** Survival of mice inoculated with Sofjin-IC-pt, Oshima-IC-pt
553 and the chimeric viruses. Mice were inoculated subcutaneously with 1,000 pfu Sofjin-IC-pt (closed
554 square), Oshima-IC/sofjin NS2A^{C} (closed triangle), Oshima-IC /sofjin NS3^{N} (open triangle),
555 Oshima-IC/sofjin NS3^{C} (closed diamond), Oshima-IC/sofjin NS4B^{N} (open diamond), or
556 Oshima-IC-pt (closed circle).
557
558
559 **Fig.4** Effect of substitutions of TBEV amino acids in NS5 and replacement of the 3'-UTR. (a)
560 Schematic representation of the genome of recombinant viruses. Single-amino acid substitutions
561 were introduced at NS5 positions 778 (NS5-778L), 827 (NS5-827S), 832 (NS5-832A), and 862
562 (NS5-862K) of Oshima-IC. The gray and white arrowheads indicate amino acids derived from
563 Sofjin-IC-pt and Oshima-IC-pt, respectively. Oshima-IC/sofjin3'-UTR_vari and
564 Oshima-IC/sofjin3'-UTR_core are Oshima-IC chimeric viruses in which the variable region and core
565 element of the 3'-UTR were replaced with those of Sofjin-IC. The gray lines indicate the regions
566 derived from the 3'-UTR of Sofjin-IC. The broken line indicates the region lacking in Sofjin-IC-pt.
567 **(b)** Growth curve of each virus in NA cells. NA cells were infected with each virus at multiplication
568 of MOI of 1. Viral titers at each time point were determined in BHK-21 cells. The data is the average
569 of three independent experiments. * At 24h post-infection, the chimeric viruses except for
570 Oshima-IC/sofjin 3'-UTR_core showed a significant difference from Sofjin-IC-pt (P<0.05). † At 48h
571 post-infection, a significant difference was observed between Sofjin-IC-pt and the other viruses
572 (P<0.01). No significant difference between Oshima-IC-pt and each chimeric virus was observed at
573 24h and 48h post-infection. **(c)** Survival of mice inoculated with Sofjin-IC-pt, Oshima-IC-pt and the
574 chimeric viruses. Mice were inoculated subcutaneously with 1,000 pfu of Sofjin-IC-pt (closed

575 square), Oshima-IC/NS5-778L (closed triangle), Oshima-IC/NS5-827S (open triangle),
576 Oshima-IC/NS5-832A (closed diamond), Oshima-IC/NS5-862K (open diamond),
577 Oshima-IC/sofjin3'-UTR_vari (open square), Oshima-IC/sofjin3'-UTR_core (open circle), and
578 Oshima-IC-pt (closed circle).
579
580 **Fig. 5** Effects of the replacement of the variable region on viral multiplication in organs. Mice were
581 infected with 1,000 pfu of Sofjin-IC-pt, Oshima/sofjin_3'-UTR vari, and Oshima-IC-pt. Virus titers
582 in the blood (a), spleen (b), and brain (c) at the indicated days after infection were determined by
583 plaque assays. The horizontal dashed lines indicate the limits of detection for the assay (100 pfu
584 mL^{-1}). Error bars represent the SD (n=3). An asterisk (*) or dagger (†) denotes a significant
585 difference compared with Oshima-IC-pt or Sofjin-IC-pt, respectively ($P < 0.05$).
586
587 **Fig. 6** Histopathological features in the brain of mice at 9 days after subcutaneous infection. Mice
588 were infected with 10^3pfu of Oshima-IC-pt ((a), (b)) or Sofjin-IC-pt ((c), (d)) and
589 Oshima/3'-UTR_vari ((e), (f)). TBEV antigens were detected using E-protein-specific antibodies
590 (brown signal in left columns). Non-suppurative encephalitis including perivascular cuffing
591 (arrowhead) was observed in mice infected with each virus (right columns).

(a)


Sofjin-IC-pt
Oshima-IC-pt
AvrII
240
AvrII
2292
Sofjin-IC/oshimaCME
(nt 240-2291)
Oshima-IC /sofjinCME
(nt 240-2291)
C
prM
E
NS1
2A
2B
3
4A
4B
5

(b)


virus titer (log_{10}pfu ml^{-1})
hours post-infection
*
†
Sofjin-IC-pt
Sofjin-IC/oshimaCME
Oshima-IC/sofjinCME
Oshima-IC-pt

(c)

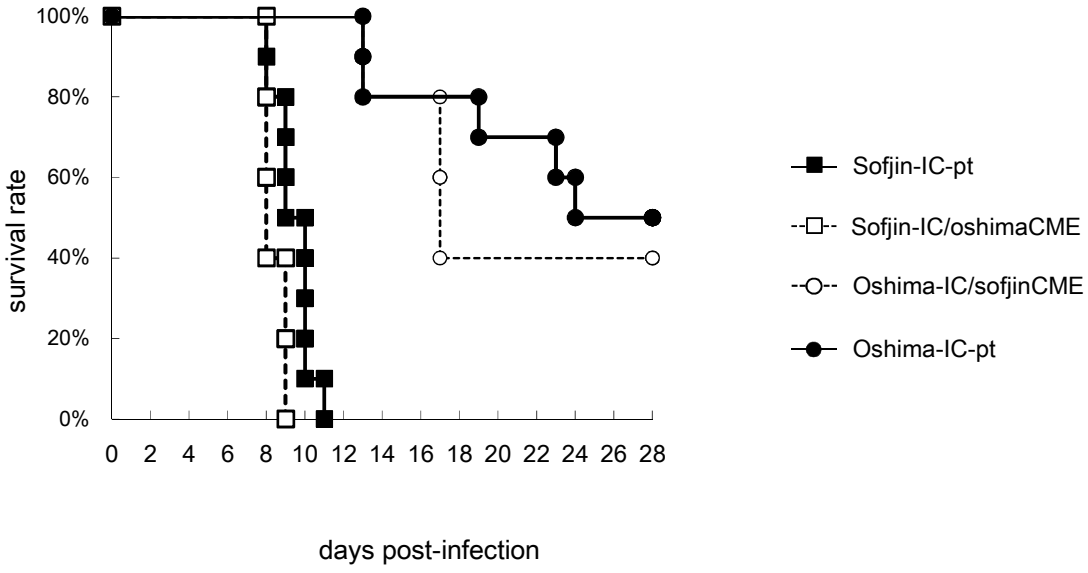
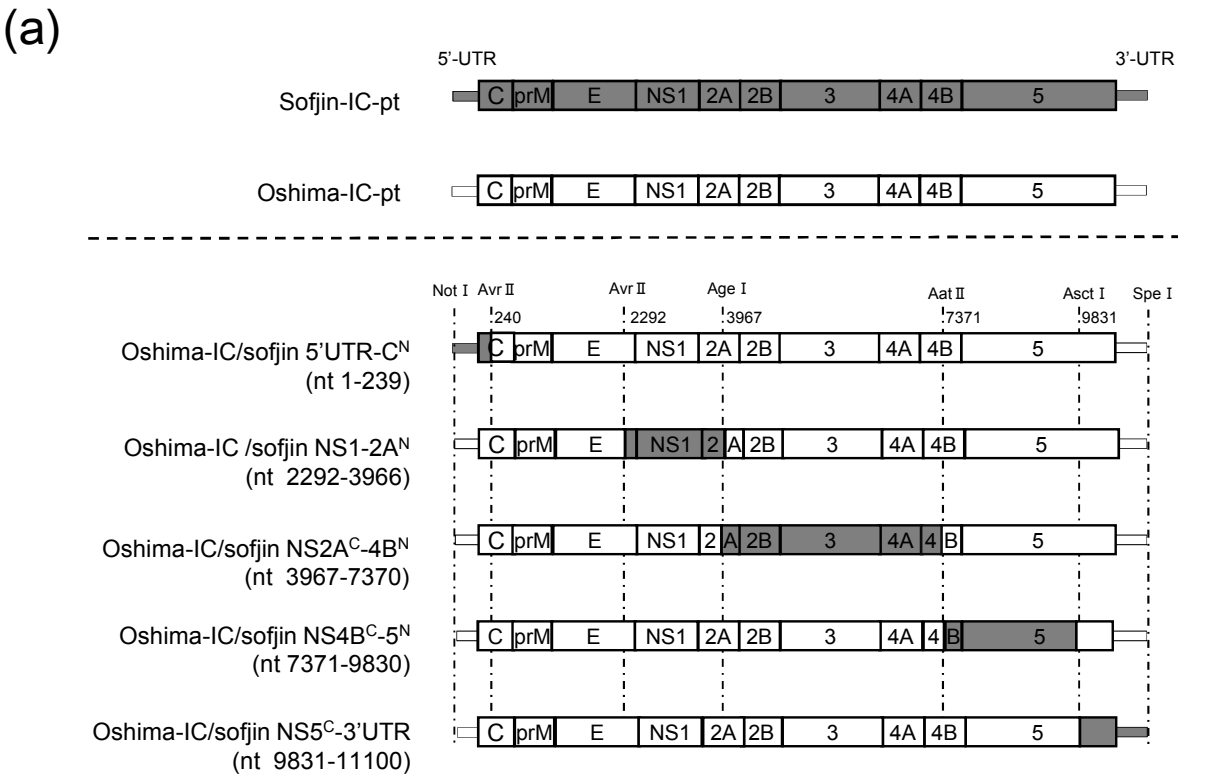
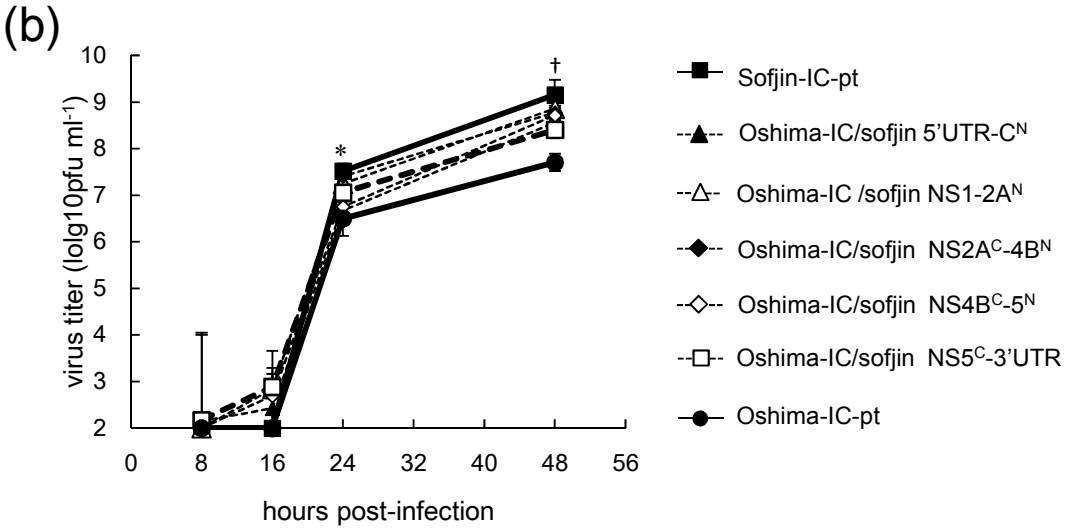

survival rate
days post-infection
Sofjin-IC-pt
Sofjin-IC/oshimaCME
Oshima-IC/sofjinCME
Oshima-IC-pt

Fig.1


(a)
5'-UTR
3'-UTR
Sofjin-IC-pt
Oshima-IC-pt
C prM E NS1 2A 2B 3 4A 4B 5
Not I
Avr II
240
Avr II
2292
Age I
3967
Aat II
7371
Asct I
9831
Spe I
Oshima-IC/sofjin 5'UTR-C^N (nt 1-239)
Oshima-IC /sofjin NS1-2A^N (nt 2292-3966)
Oshima-IC/sofjin NS2A^C-4B^N (nt 3967-7370)
Oshima-IC/sofjin NS4B^C-5^N (nt 7371-9830)
Oshima-IC/sofjin NS5^C-3'UTR (nt 9831-11100)


(b)
virus titer (lolg10pfu ml-1)
hours post-infection
*
†
Sofjin-IC-pt
Oshima-IC/sofjin 5'UTR-C^N
Oshima-IC /sofjin NS1-2A^N
Oshima-IC/sofjin NS2A^C-4B^N
Oshima-IC/sofjin NS4B^C-5^N
Oshima-IC/sofjin NS5^C-3'UTR
Oshima-IC-pt

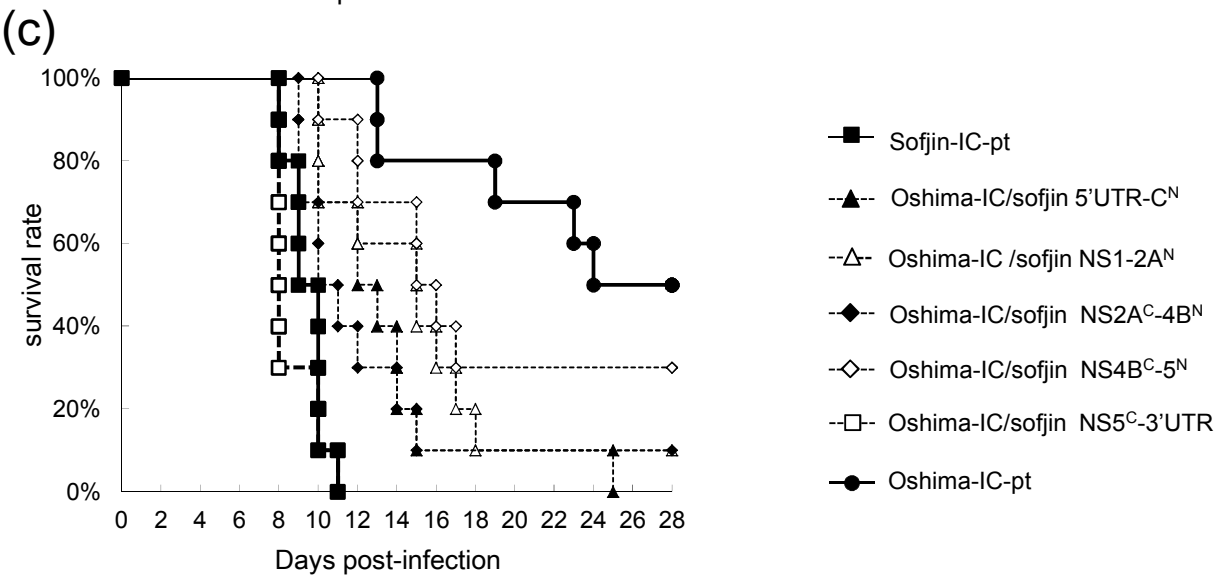
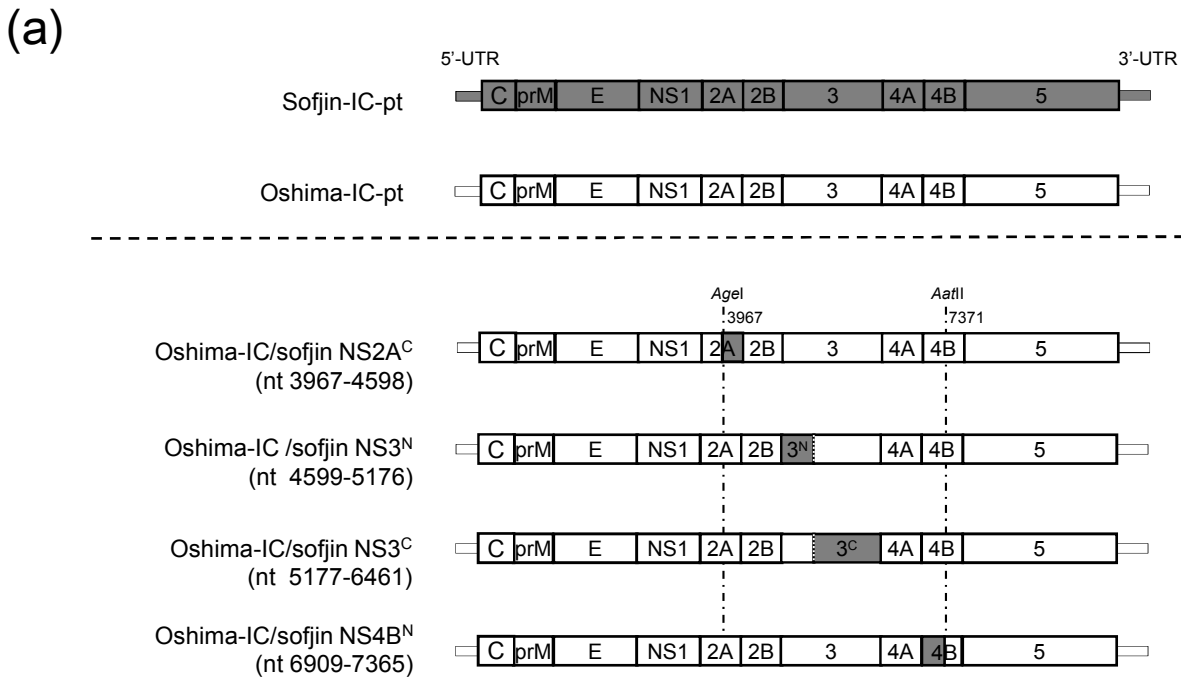
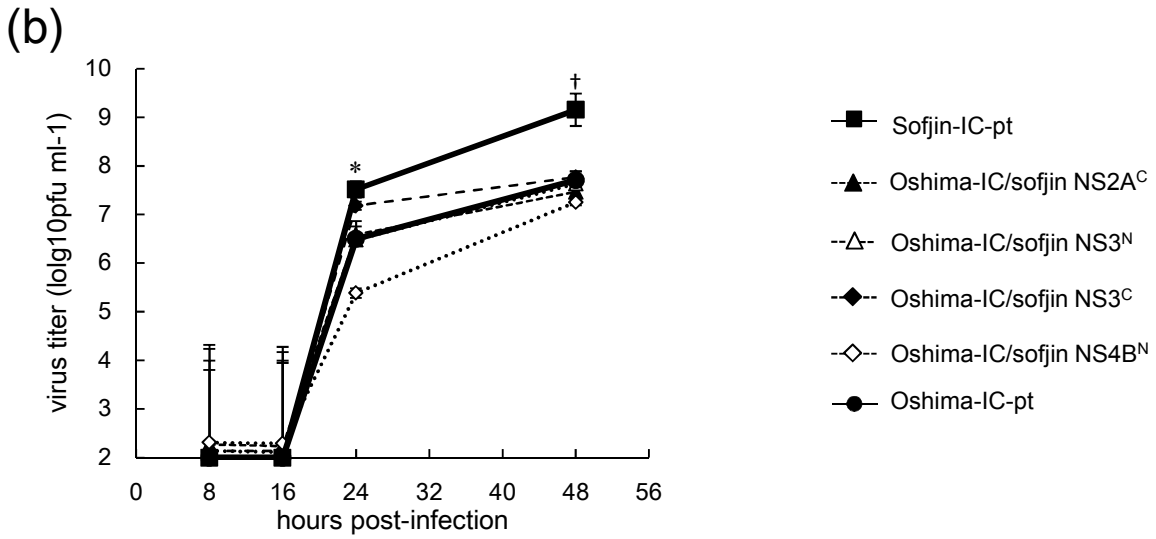

(c)
survival rate
Days post-infection
Sofjin-IC-pt
Oshima-IC/sofjin 5'UTR-C^N
Oshima-IC /sofjin NS1-2A^N
Oshima-IC/sofjin NS2A^C-4B^N
Oshima-IC/sofjin NS4B^C-5^N
Oshima-IC/sofjin NS5^C-3'UTR
Oshima-IC-pt

Fig.2


(a)
5'-UTR
3'-UTR
Sofjin-IC-pt
Oshima-IC-pt
C prM E NS1 2A 2B 3 4A 4B 5
AgeI 3967
AatII 7371
Oshima-IC/sofjin NS2A^C (nt 3967-4598)
Oshima-IC /sofjin NS3^N (nt 4599-5176)
Oshima-IC/sofjin NS3^C (nt 5177-6461)
Oshima-IC/sofjin NS4B^N (nt 6909-7365)


(b)
virus titer (lolg10pfu ml-1)
hours post-infection
Sofjin-IC-pt
Oshima-IC/sofjin NS2A^C
Oshima-IC/sofjin NS3^N
Oshima-IC/sofjin NS3^C
Oshima-IC/sofjin NS4B^N
Oshima-IC-pt

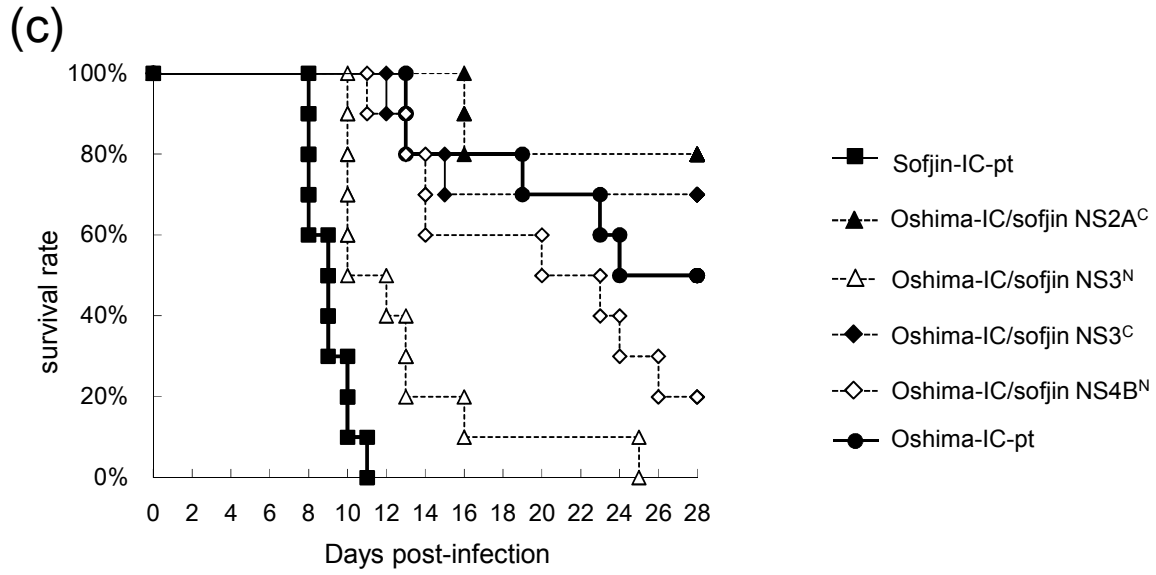

(c)
survival rate
Days post-infection
Sofjin-IC-pt
Oshima-IC/sofjin NS2A^C
Oshima-IC/sofjin NS3^N
Oshima-IC/sofjin NS3^C
Oshima-IC/sofjin NS4B^N
Oshima-IC-pt

Fig.3

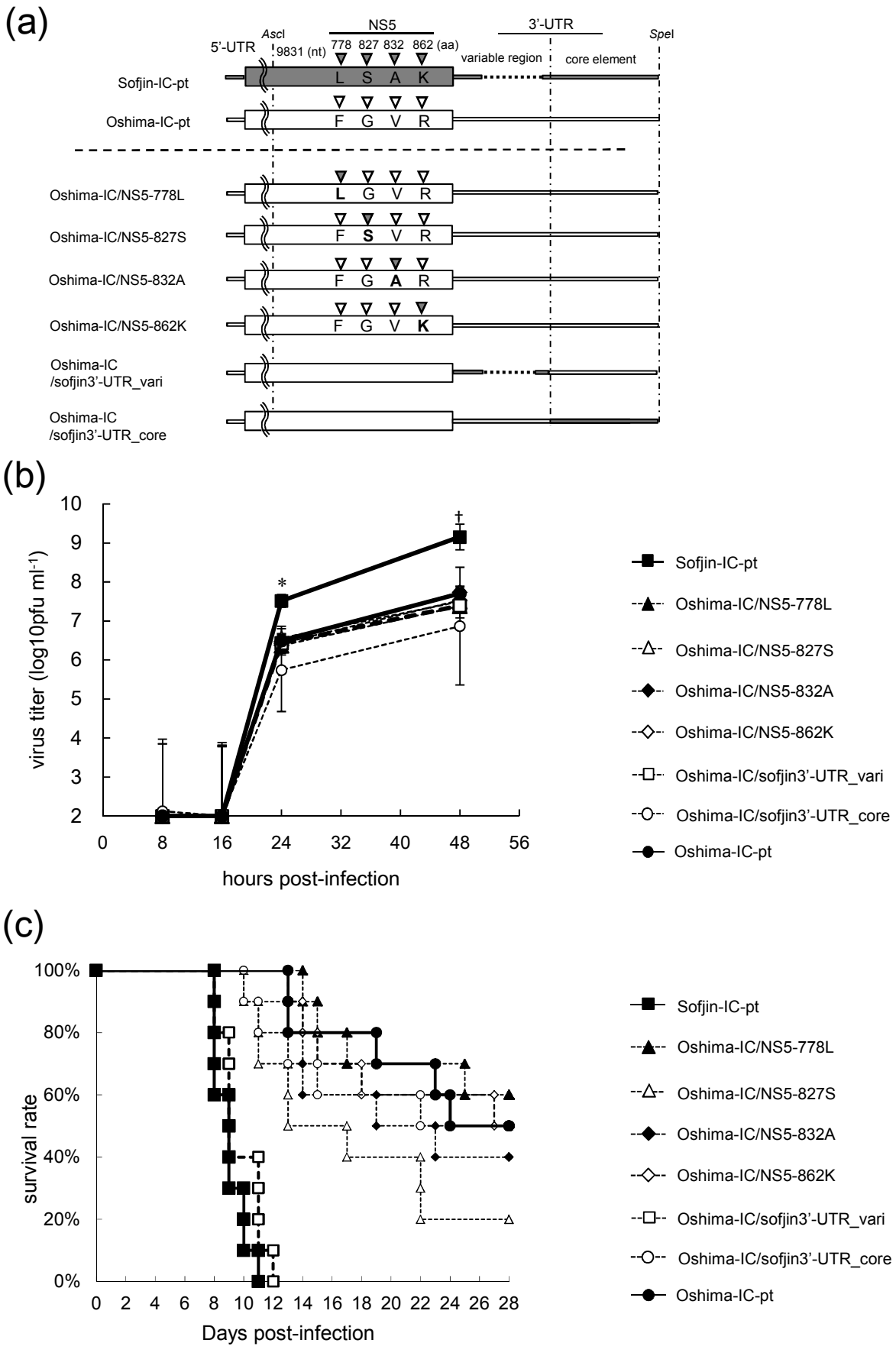

(a)
5'-UTR
AscI
9831 (nt)
NS5
778 827 832 862 (aa)
variable region
3'-UTR
core element
SpeI
Sofjin-IC-pt
L S A K
Oshima-IC-pt
F G V R
Oshima-IC/NS5-778L
L G V R
Oshima-IC/NS5-827S
F S V R
Oshima-IC/NS5-832A
F G A R
Oshima-IC/NS5-862K
F G V K
Oshima-IC /sofjin3'-UTR_vari
Oshima-IC /sofjin3'-UTR_core
(b)
virus titer (log10pfu ml-1)
hours post-infection
Sofjin-IC-pt
Oshima-IC/NS5-778L
Oshima-IC/NS5-827S
Oshima-IC/NS5-832A
Oshima-IC/NS5-862K
Oshima-IC/sofjin3'-UTR_vari
Oshima-IC/sofjin3'-UTR_core
Oshima-IC-pt
(c)
survival rate
Days post-infection
Sofjin-IC-pt
Oshima-IC/NS5-778L
Oshima-IC/NS5-827S
Oshima-IC/NS5-832A
Oshima-IC/NS5-862K
Oshima-IC/sofjin3'-UTR_vari
Oshima-IC/sofjin3'-UTR_core
Oshima-IC-pt

Fig.4

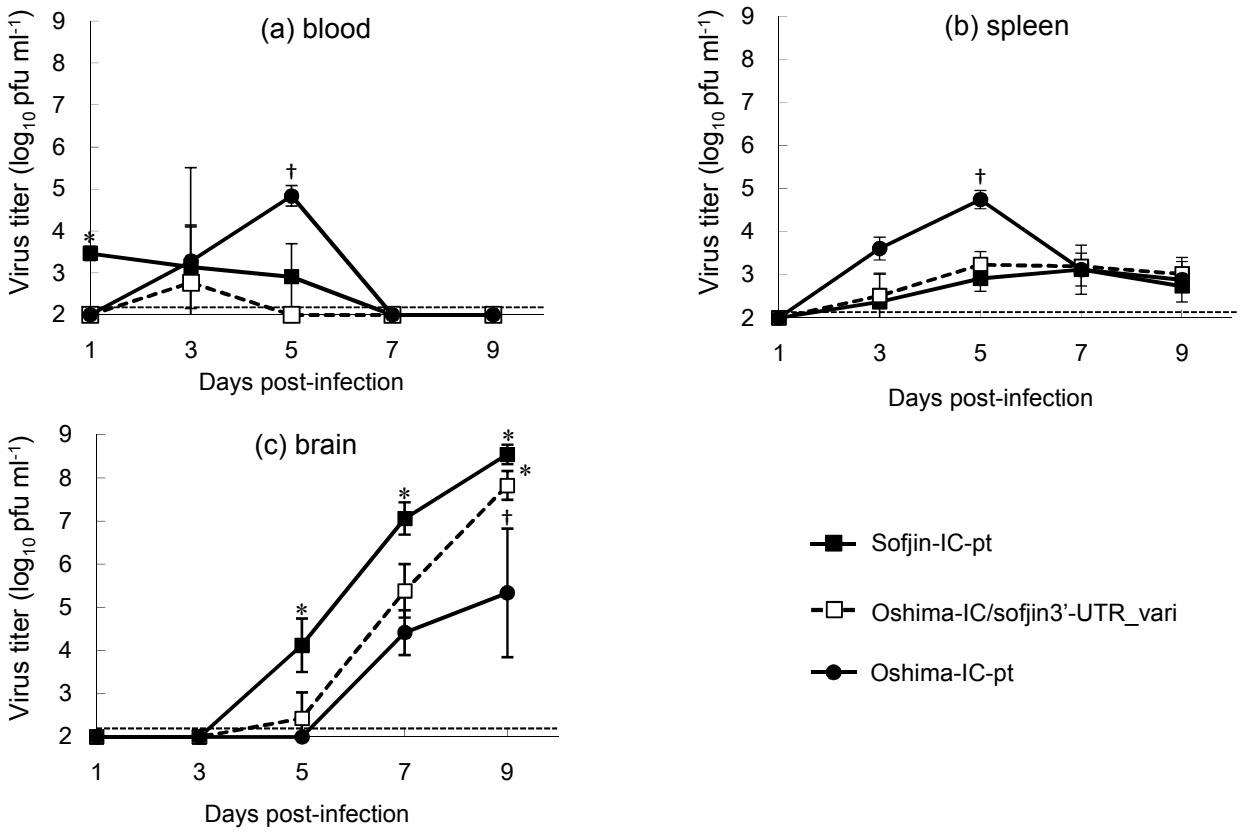

(a) blood
Virus titer (log_{10} pfu ml^{-1})
Days post-infection
(b) spleen
Virus titer (log_{10} pfu ml^{-1})
Days post-infection
(c) brain
Virus titer (log_{10} pfu ml^{-1})
Days post-infection
Sofjin-IC-pt
Oshima-IC/sofjin3'-UTR_vari
Oshima-IC-pt

Fig.5

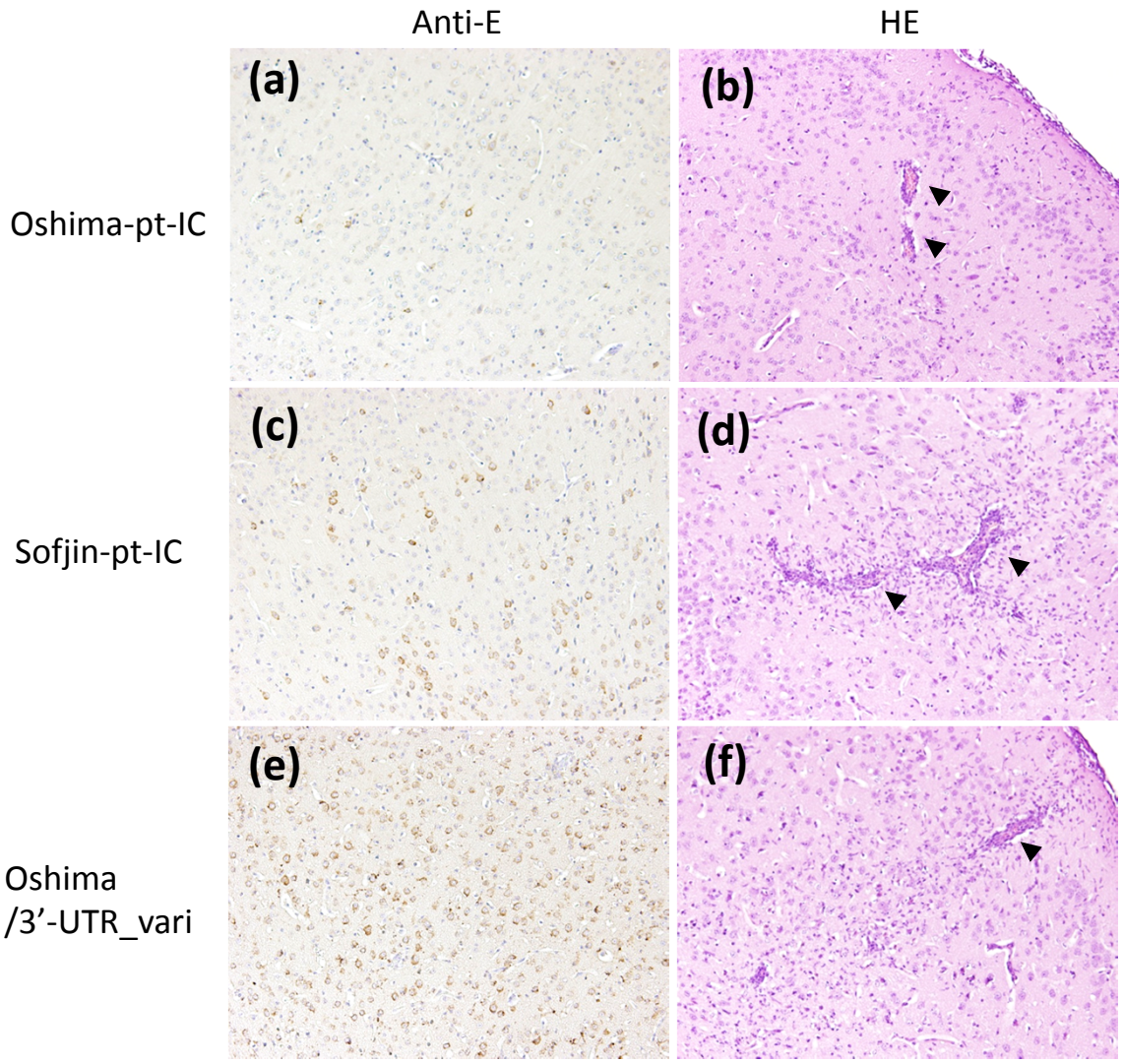


Fig.6