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Association of toll-like receptor 3 gene polymorphism with subacute sclerosing panencephalitis

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Innate immunity plays an important role in measles virus (MV) infection. MV-derived double-stranded RNA is recognized by toll-like receptor 3 (TLR3), retinoic acid–inducible protein I (RIG-I) and melanoma differentiation–associated gene 5 (MDA5). We investigated whether genes encoding these molecules contributed to the development of subacute sclerosing panencephalitis (SSPE) in Japanese individuals. Four single nucleotide polymorphisms (SNPs) of the three genes (*TLR3* rs3775291:Leu412Phe, *RIG1* rs277729 and rs9695310, and *MDA5* rs4664463) were assessed in 40 SSPE patients and 84 controls. Because the *TLR3* SNP showed a positive association with SSPE, three additional SNPs were subjected to haplotype analysis. The frequency of 412Phe allele of *TLR3* rs3775291 in SSPE patients was significantly higher than that in controls ($P = .03$). In haplotype analysis of four SNPs in the *TLR3* gene, the frequency of −7C/IVS3+71C/Phe412/c.1377C haplotype was significantly increased in SSPE patients ($P = .006$, odds ration [OR]: 2.2). *TLR3* gene may confer host genetic susceptibility to SSPE in Japanese individuals. -
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Introduction

Subacute sclerosing panencephalitis (SSPE) is a fatal degenerative neuronal disease caused by persistent infection with a measles virus (MV), the nature of which is quite different from that of common wild types. The viruses isolated from SSPE brains, called SSPE viruses, are not able to form infective particles owing to extensive mutations in their envelope-associated proteins, especially the M protein. Sequence analysis, however, has revealed that most of the mutations considered to be common and specific for SSPE viruses are also found in recently isolated wild-type strains (Jin *et al*, 2002; Kai *et al*, 1996)

In addition to viral factors, host factors seem to contribute to the development of SSPE (Gascon, 1996; Schneider-Schaulies *et al*, 1999). Epidemiologic studies have shown that contraction of measles before 2 years of age increases the risk of SSPE, suggesting that immaturity of the host immune system and central nervous system (CNS) plays an important role in host susceptibility factors for the development of SSPE. Our previous study demonstrated that the combination of TT genotype of

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interleukin-4 gene promoter −589C/T single-nucleotide polymorphism (SNP) and an allele of interferon regulatory factor 1 (IRF-1) gene GT repeat polymorphism, and the T allele of −88 G/T SNP of *MxA* gene encoding an antivirally active protein, MxA, were more frequent in SSPE patients than in controls (Inoue *et al*, 2002; Torisu *et al*, 2004).

Innate immunity is responsible for the detection of and the initial protective response to viral infections (Kawai and Akira, 2006). Specific receptors of the innate immune system play a key role in detecting the presence of viruses. In MV infection, viral double-stranded (ds) RNA is formed during the replication cycle and recognized by toll-like receptor 3 (TLR3) (Tanabe *et al*, 2003). Other cytosolic receptors for dsRNA including retinoic-acid-inducible protein I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) are also involved in antiviral response to MV infection (Berghall *et al*, 2006; Plumet *et al*, 2007). Recognition of dsRNA derived from MV triggers the production of type I interferon (IFN), which plays an important role in MV clearance.

It has been reported that common mutations of genes encoding molecules related to innate immunity influences susceptibility to viral infections. *TLR4* mutations were associated with an increased risk of severe respiratory syncytial virus bronchiolitis (Tal *et al*, 2004). Rapid progression of HIV-1 infection was associated with *TLR9* polymorphisms (Bochud *et al*, 2007). To determine the role of *TLR3*, *RIG1*, and *MDA5* genes as a host genetic factor for the development of SSPE, we have performed an association study on SNPs of these genes in the Japanese SSPE patients and controls.

Results

The frequencies of each allele of tag SNPs of *TLR3* L412F (rs3775291*)*, *RIG1* rs277729, and *MDA5* rs4664463, and of three additional SNPs of *TLR3* (−7C/A; rs3775296, IVS3+71C/A; and c.1377C/T; rs3775290) for haplotype analysis in SSPE patients and controls are shown in Table 1. The distribution of the genotypes in the controls was in Hardy-Weinberg equilibrium. The frequency of *TLR3* 412Phe allele (rs3775291) in SSPE patients was significantly higher than that in controls ($P = .03$). There were no significant differences in allele frequencies of SNPs of *RIG1* and *MDA5* genes between SSPE patients and controls.

Table 2 shows linkage disequilibrium (LD) analysis of the four SNPs spanning 6.5 kb of *TLR3*. A significant linkage disequilibrium was observed among them. To investigate if a particular haplotype constituted by the four SNPs was associated with SSPE, haplotype frequencies were estimated and association analysis was done (Table 3). We observed only one haplotype containing 412Phe allele. The frequency of −7C/IVS3+71C/Phe412/c.1377C haplotype, the sole haplotype containing 412Phe allele, was significantly higher in SSPE patients than in controls ($P = .006$, odds ratio: 2.2, confidence interval: 1.3–3.9).

Discussion

In the current study, we first demonstrated an association between a SNP of *TLR3* and SSPE in Japanese population. These data suggest that 412Phe allele of *TLR3* and the haplotype containing this allele may be host genetic factors that confer a predisposition to SSPE. These results imply that TLR3 may participate in the pathogenesis of SSPE.

TLR3 is expressed in conventional dendritic cells (DCs), a variety of epithelial cells including airway, genital tract, biliary and intestinal epithelial cells, and in the brain (Rock *et al*, 1998). TLR3 is localized primarily in endosomal membrane and recognizes virus-derived dsRNA. TLR3 signaling activates the transcription factor-κB (NF-κB) and IRF-3. IRF-3

Table 1 Allele frequencies of each polymorphism

Gene	Polymorphism	Allele	Control (%)	SSPE (%)	*P* value
RIG1	rs277729	G	120 (71.4)	50 (62.5)	.16
		T	48 (28.6)	30 (37.5)	
	rs9695310	C	118 (70.2)	54 (67.5)	.66
		G	50 (29.8)	26 (32.5)	
MDA5	rs4664463	T	137 (81.5)	70 (87.5)	.24
		C	31 (18.5)	10 (12.5)	
TLR3	−7C/A	C	130 (77.4)	60 (75.0)	.68
		A	38 (22.6)	20 (25.0)	
	IVS3+71C/A	C	132 (78.6)	61 (76.3)	.68
		A	36 (21.4)	19 (23.8)	
	c.1243C/T	C (L)	122 (72.6)	47 (58.8)	.03
	(L412F)	T (F)	46 (27.4)	33 (41.3)	
	c.1377C/T	C	107 (63.7)	53 (66.3)	.69
		T	61 (36.3)	27 (33.8)	

Note. P values were calculated using 2×2 chi-square test.

Table 2 Linkage disequilibrium between all pairs of loci (D') in *TLR3* gene

	IVS3+71C/A	Leu412Phe	c.1377C/T
−7C/A	0.83	0.77	0.84
IVS3+71C/A		0.88	0.90
Leu412Phe			1

induces expression of type I interferons (IFNs), which contribute to antiviral effect via activation of other genes, including 2′-5′-oligoadenylate synthetase and M×A genes (Matsumoto *et al*, 2004). In the brain, both neurons and glial cells have been shown to express TLR3 and initiate inflammatory and antiviral responses upon being triggered with dsRNA (Lafon *et al*, 2006; Town *et al*, 2006), whereas neither RIG-I nor MDA5 is expressed in the brain (Daffis *et al*, 2007; DiSepio *et al*, 1998; Kang *et al*, 2004).

Studies using TLR3-deficient mice showed that TLR3 played contradictory roles in viral infection and general outcome depended on several factors, such as the type of virus, the cell type that is infected, and the stage of infection (Vercammen *et al*, 2008). TLR3-deficient mice had resistance to influenza A virus and West Nile virus (WNV) infections due to the absence of TLR3-mediated inflammatory signaling (Le Goffic *et al*, 2006; Wang *et al*, 2004), whereas they showed normal resistance to lymphocytic choriomeningitis virus, vesicular stomatitis virus, and reovirus (Edelmann *et al*, 2004), and were susceptible to encephalomyocarditis virus and mouse cytomegalovirus (Hardarson *et al*, 2007; Tabeta *et al*, 2004). The association between genetic variation of *TLR3* and human diseases has been suggested. Polymorphisms in the *TLR3* gene may be associated with type 1 diabetes, Stevens-Johnson syndrome, and toxic epidermal necrolysis (Pirie *et al*, 2005; Ueta *et al*, 2007). Heterozygous variant for Leu412Phe of the *TLR3* gene were associated with low antibody and lymphoproliferative responses to measles vaccination, suggesting that the SNP influenced modulation of the immune response to measles vaccine (Dhiman *et al*, 2008). Recently, Zhang *et al* identified a dominant-negative *TLR3* allele in patients with herpes simplex virus 1 (HSV-1) encephalitis (Zhang *et al*, 2007). A heterozygous *TLR3* mutation (Phe554Ser) was associated with impaired TLR3-dependent induction of IFNs in response to HSV-1. 412Leu is located near the concave surface of the TLR3 ectodomain, which binds directly to short dsRNA (Bell *et al*, 2006). Based on the analysis of the crystal structure of TLR3 ectodomain, it was predicted that 412Phe would destabilize the solenoid structure and might disrupt potential glycosylation of neighboring residue Asn413, which was observed to have *N*-acetylglucosamines attached (Bell *et al*, 2005; Ranjith-Kumar *et al*, 2007). By a reporter assay of TLR3-dependent activation, 412Phe allele of *TLR3* gene showed reduced IFN production in response to a synthetic dsRNA (Ranjith-Kumar *et al*, 2007). Accordingly, it is likely that 412Phe allele is associated with reduced ability to control viral infection as Phe554Ser mutation in HSV-1 encephalitis patients.

MV seems to gain access to the CNS at the time of primary infection, as the MV genome was detected on reverse transcription polymerase chain reaction (PCR) in cerebrospinal fluid (CSF) samples not only from patients with measles encephalitis and SSPE but also from those with acute measles (Nakayama *et al*, 1995). In SSPE brains, neurons, oligodendrocytes, astrocytes, and microvascular endothelial cells have been found to be infected (Allen *et al*, 1996). Microglia and astrocytes are endogenous cells of the CNS that are key players in the immune responses that occur within this compartment (Becher *et al*, 2000). MV infection induces type I IFN via TLR3 and type I IFN dramatically up-regulates TLR3 expression in a positive-feedback fashion in virus-infected cells (Tanabe *et al*, 2003). Previous studies have shown that type I IFN acts as a barrier to efficient MV replication in mice (Mrkic *et al*, 1998). Although the role of TLR3 signaling in MV infection in the brain has not been evaluated in TLR3-deficient mice, it is likely that 412Phe allele of *TLR3* gene with reduced TLR3-dependent IFN production or haplotype including this allele may be associated with high levels of viral invasion and replication in the brain in primary MV infection. Initial high MV titer in the brain would contribute to the establishment of persistent CNS infection, an essential step in the pathogenesis of SSPE. In our previous study, we reported an association of *M×A* promoter −88T allele with a high MxA-producing capability and SSPE. It is suggested that, once

Table 3 Estimated haplotype frequencies in healthy controls and SSPE patients

Haplotype	Controls	SSPE	*P* value	OR (95%CI)
−7C/IVS3+71C/Leu412/c.1377C	0.341	0.25	.15	0.6 (0.4∼1.2)
−7C/IVS3+71C/Phe412/c.1377C	0.241	0.412	.006*	2.2 (1.3∼3.9)
−7A/IVS3+71A/Leu412/c.1377T	0.162	0.237	.16	1.6 (0.8∼3.2)
−7C/IVS3+71C/Leu412/c.1377T	0.147	0.067	.19	0.6 (0.2∼1.3)

Note. OR: odds ratio, CI: confidence interval.
The frequencies were estimated using the EH software program.
Haplotype with frequency >0.05 were shown.
*Corrected *P* value was .024.

persistent CNS infection has established, MV might benefit from IFN signaling through attenuation of viral gene expression and resultant escape from immunologic clearance. We could not find any synergism of Leu412Phe of *TLR3* gene and −88G/T of *MxA* gene (data not shown).

In summary, among SNPs of the genes encoding molecules related to dsRNA recognition, frequencies of 412Phe allele of *TLR3* gene and haplotype including it were significantly higher in SSPE patients. These findings suggested that *TLR3* might confer host genetic susceptibility to SSPE in Japanese population.

Materials and methods

Subjects
The study population comprised 40 unrelated SSPE patients (27 males and 13 females) and 84 unrelated normal school children. All the SSPE patients fulfilled the diagnostic criteria, that is, clinical features, increased MV antibody titer in the CSF, and typical electroencephalograph (EEG) showing periodic slow wave complexes early in the disease. The age at onset of SSPE ranged between 2 and 15 years (mean ± SD, 8.0 ± 3.1 years). Thirty-five patients had contracted natural measles occurring between ages 0.4 and 4 years (1.3 ± 0.9 years). The history of natural measles was unknown in the other five patients, including one with a history of live attenuated measles vaccination. The places where SSPE patients lived and had contracted measles were distributed all over Japan. The age of SSPE patients, when studied, ranged between 5 and 26 years (median 11 years, mean ± SD 12.4 ± 5.1 years). The control subjects were randomly selected from among normal schoolchildren around 10 years of age and were not matched for age or histories of measles and measles vaccination. Informed consent was obtained from their parents. The current study was approved by the Ethics Committee of Kyushu University, Japan.

DNA extraction
Genomic DNA was extracted from peripheral blood using a QIAmp DNA Blood Kit (Qiagen, Tokyo, Japan).

Selection of SNPs
We used data on common SNPs from HapMap (Japanese samples, October 2005 release) to identify tag SNPs. We selected one tag SNPs with the highest minor allele frequency within one linkage disequilibrium block: *TLR3* L412F (rs3775291), *RIG1* rs277729 and rs9695310, and *MDA5* rs4664463. We selected two tag SNPs of *RIG1* gene, because this gene consists of two LD blocks. For haplotype analysis, we selected additional three SNPs of *TLR3* gene based on the previous study; −7C/A (rs3775296), IVS3 + 71C/A, and c.1377C/T (rs3775290) (Noguchi *et al*, 2004).

Genotype analysis of SNPs in the TLR3, RIG1, and MDA5 genes
SNPs and genotyping methods are described in Table 4. Genotyping of each subject was performed by either TaqMan method or PCR restriction fragment length polymorphism (RFLP). TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA) was performed following the manufacturer's instructions. PCR was carried out with mixes consisting of 8 ng of genomic DNA, 5 μl of Taqman master mix, 0.5 μl of 20 × assay mix, and double distilled H_2O up to 10 μl of final volume. The following amplification protocol was used: denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 92°C for 15 s and annealing and extension at 60°C for 1 min. After PCR, the genotype of each sample was attributed automatically by measuring the allelic specific fluorescence on the ABI PRISM 7700 Sequence Detection Systems using the SDS 2.2.2 software for allelic discrimination (Applied Biosystems). Additional three SNPs of *TLR3* gene were analyzed by PCR-RFLP as previously described (Noguchi *et al*, 2004). PCR was performed with a listed primer pair under the following conditions: 94°C for 5 min; then 35 cycles of 94°C for 30 s, 56°C for 1 min, and 72°C for 1min. The PCR products were digested with each restriction enzyme, followed by separation in 3%

Table 4 Genotyping methods of each polymorphism

Gene	Polymorphism	Method	Primer	Enzyme
TLR3	−7C/A (rs3775296)	RFLP	5'-gcatttgaaagccatctgct 5'-aagttggcggctggtaatct	*MboII*
	IVS3 + 71C/A	RFLP	5'-gctggaaaatctccaagagc 5'-gaggctagagagcattacattcat	*NlaIII*
	L412F (rs3775291)	TaqMan		
	c.1377 C/T (rs3775290)	RFLP	5'-ccaggcataaaaagcaatatg 5'-ggaccaaggcaaaggagttc	*TaqI*
RIG1	rs277729	TaqMan		
	rs9695310	TaqMan		
MDA5	rs4664463	TaqMan		

agarose gels and visualization by ethidium bromide staining.

Haplotype analysis of SNPs in the TLR3 gene
We performed haplotype analysis using four SNPs in the *TLR3* gene. Haplotype frequencies were estimated using the Estimating Haplotype Frequencies (EH) software program (ftp://linkage.rockefeller.edu/software/rh). LD coefficients D' values, odd's ratio, and 95% were calculated using data in the EH software program.

Statistical analyses
The Hardy-Weinberg equilibrium of alleles in controls was assessed by means of 2×2 χ^2 statistics. Differences in the allele or genotype frequencies between two groups were evaluated by means of the χ^2 analysis with a 2×2 or 2×3 contingency table. A P value of .05 was considered to be significant except for haplotype analysis, in which a P value of .05/4 (= .0125) was considered to be significant, taking Bonferroni's multiple adjustments into consideration.

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