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Gene expression profiles in peripheral blood mononuclear cells from patients with subacute sclerosing panencephalitis using oligonucleotide microarrays

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To investigate the molecular basis for measles virus persistence in patients with subacute sclerosing panencephalitis (SSPE), the authors used a high-density oligonucleotide microarray, and found that the expression of *granulysin* in peripheral blood mononuclear cells was significantly lower in the patients than in the controls. By a quantitative reverse transcriptase–polymerase chain reaction, the mRNA levels of *granulysin* were decreased in 30 SSPE patients, and were increased in 7 measles patients, as compared to the 23 controls. These results imply that *granulysin* might play a role in the host defense against measles virus and possibly be involved in the pathogenesis or pathophysiology of SSPE. *Journal of NeuroVirology* (2005) **11,** 299–305.

Keywords: granulysin; measles; oligonucleotide microarray; polymorphism; SSPE

Introduction

Subacute sclerosing panencephalitis (SSPE) is a slowly progressive central nervous system (CNS) complication of measles. Both viral and host factors appear to be involved in the pathogenesis of SSPE (Gascon, 1996; Schneider-Schaulies *et al*, 1999, 2003). As viral factors for the development of SSPE, measles viruses (MVs) isolated from the CNS of SSPE patients are replication defective and have extensive mutations within the envelope-associated genes. However, matrix gene mutations, which are thought to be characteristic of SSPE viruses, have also been found in recent clinical isolates (Kai *et al*, 1996). Regarding host factors, epidemiological studies have shown that the contraction of measles before 2 years of age increases the risk of developing SSPE, thus suggesting that the immaturity of the host immune system and CNS themselves may play a role in the development of SSPE (Gascon, 1996; Schneider-Schaulies *et al*, 1999, 2003). In our previous studies (Kusuhara *et al*, 2000; Inoue *et al*, 2002; Torisu *et al*, 2004), we demonstrated that the genotype combination of *interleukin-4* promoter −589 TT and

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an allele of *interferon regulatory factor-1* gene GT repeat polymorphism was more frequent in SSPE patients than in controls, suggesting that the genetic predisposition to T helper (Th) 2 dominance in SSPE patients might play a role in the persistence of MV through a reduction in the Th1 response. Furthermore, a genetic analysis of the antivirally active MxA protein revealed that homozygotes of the −88T allele were more frequently seen in SSPE patients than in the controls.

MV has tropism for monocytic, lymphocytic, and neural cells. Seventy to 90% of peripheral blood mononuclear cells (PBMCs) from patients with SSPE contain MV RNA sequences (Fournier *et al*, 1985) and MV RNA was also detected in an appendix removed before the onset of SSPE (Fournier *et al*, 1986), although the contradictory findings were also reported (Schneider-Schaulies *et al*, 1991). In addition, recent studies demonstrated the afferent and efferent mechanisms of immune cells into and out of the CNS, respectively (Ransohoff *et al*, 2003). Thus, certain mechanisms should exist to permit a widespread persistent MV infection not only in the CNS but also in the immune system in SSPE patients. In fact, an impairment of MV-specific cell-mediated immunity and a decreased Th-1 cytokine production were observed in a significant proportion of SSPE patients (Ewan and Lachmann, 1977; Dhib-Jalbut *et al*, 1989; Hara *et al*, 2000), although no universal defect has yet been identified in the immune system. Recent advances in the studies of gene expression profiling of blood cells using microarray technology have provided new insights into the pathogenesis of such disorders as cancers and autoimmune diseases (Brutsche *et al*, 2001; Rus *et al*, 2002). In the present study, to investigate the molecular basis of SSPE, we analyzed the difference in the gene expression of PBMCs between SSPE patients and controls using a high-density oligonucleotide microarray containing approximately 30,000 human genes. Next, a quantitative reverse transcriptase–polymerase chain reaction (RT–PCR) was carried out to validate the microarray results. In addition, we also performed an association study on the polymorphisms of a gene of interest in the SSPE patients and controls.

Results

Among approximately 30,000 genes analyzed by an oligonucleotide microarray, 12,121 genes showed reliable expression levels in PBMCs from the SSPE patients and controls. There were 27 genes with statistically significant differences in the expression between the two groups according to Welch *t* test (*P* value cut-off .01). All 27 genes are listed in Table 1. The genes with mean expression levels demonstrating over two-fold differences between the SSPE patients and controls are also listed in Table 1. *Granulysin* (*GNLY*, MIM188855) was the only common gene included in the two lists and its average expression ratio of SSPE to normal was 0.458 (range 0.453 to 0.463). Contrary to our expectations, no immunity-related genes included among the 27 genes in the table, except for *GNLY*.

To confirm the expression data from the oligonucleotide microarray analysis and to extend the analysis to a larger set of clinical samples, we analyzed the expression levels of the two isoforms of *GNLY* in 30 patients with SSPE, 23 normal controls, and 7 patients with measles by quantitative RT-PCR. Figure 1 shows the relative expression levels of granulysin isoforms 519 (Figure 1A) and 520 (Figure 1B). The relative expression levels of the two granulysin isoforms were significantly different among the normal, SSPE, and measle groups ($P < .001$).

The relative expression levels of 519 and 520 tended to be lower in the PBMCs from the SSPE patients than in those from the normal controls (*P* value .025 by two sided Mann-Whitney *U* test and *P* value .038 by two sided Welch's *t* test, respectively). The relative expression levels of 519 tended to be higher in the measles group than in the normal control group (*P* value .039 by two-sided Mann-Whitney *U* test). However, these differences were not significant when Bonferroni correction in multiple comparisons was applied. The relative expression levels of 520 were significantly higher in the measles group than in the

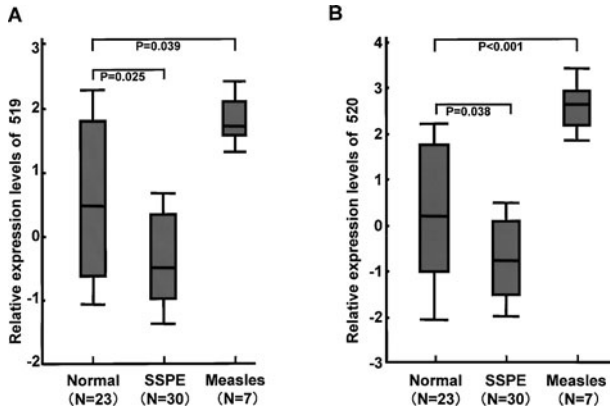

Figure 1 Expression levels of *GNLY* in PBMCs from normal controls, SSPE patients, and measles patients. Box and whisker plots show the 10th, 25th, 50th (median), 75th, and 90th percentile values for the 519 (**A**) and 520 (**B**) mRNA expressions in PBMCs from the normal controls, SSPE patients, and measles patients. Log-transformed values of the expression ratios to rRNA are shown. The values in parentheses indicate the number of the samples. The log-transformed medians of 519 mRNA expressions were 0.46 (range −1.6 to 3.5) in the normal control group, −0.52 (−2.5 to 2.0) in the SSPE group, and 1.7 (0.93 to 2.6) in the measles group. The medians of 520 mRNA expressions were 0.19 (−2.8 to 3.1) in the normal control group, −0.74 (−3.0 to 1.7) in the SSPE group, and 2.63 (1.8 to 4.1) in the measles group. The relative expression levels of 519 and 520 were significantly different among the three groups ($P < .001$ by Kruskal-Wallis *H* test and one-way ANOVA, respectively). The *P* values for normal versus SSPE and normal versus measles are shown. A Bonferroni multiple adjustments were made to the level of significance, which was set at P of $< .025$.

Table 1 A list of genes with different expressions between the SSPE and the normal groups

Oligo ID	*Gene symbol*	*Expression ratio*†	*Function*
Significantly up-regulated genes in SSPE*			
AGhsC171515		2.33	
AGhsB110501	LOC144097	1.78	
AGhsB181413	hspc055	1.58	
AGhsC070113		1.54	
AGhsC051013	LOC57164	1.42	
AGhsB211013	dj211d12.4	1.30	
AGhsA140622	*DDX39*	1.27	ATP-dependent helicase activity
AGhsB141014	*ARHGAP1*	1.23	RHO GTPase activator
AGhsB241313	DKFZp434H0512	1.23	
AGhsB010615	*KIAA0645*	1.15	Intracellular signaling cascade
AGhsA181309	*MTA1L1*	1.15	Transcription factor activity
AGhsB261313		1.15	
Significantly down-regulated genes in SSPE*			
AGhsA020814	*GNLY*	0.46	Cellular defense response
AGhsA051623	*SNX6*	0.59	Negative regulation of EGF receptor
AGhsA020403	*BIN2*	0.64	Endocytosis; synaptic transmission
AGhsA130712	*ATP5E*	0.74	
AGhsA210415	*TCTEL1*	0.76	
AGhsB221623	*KIAA1265*	0.80	Metal ion transport
AGhsC140322		0.83	
AGhsC060107	*OR1A1*	0.83	G-protein coupled receptor
AGhsB211414		0.83	
AGhsC141207		0.83	
AGhsB210411	*RNF29*	0.83	Signal transduction
AGhsB120923	*COTL1*	0.84	Actin-binding proteins
AGhsA091105	*CA12*	0.85	One-carbon compound metabolism
AGhsB190806		0.86	
AGhsC160710		0.87	
Over two fold differences in the mean expression levels			
AGhsA020814	*GNLY*	0.46	Cellular defense response
AGhsA061612	*ANXA2*	0.50	Osteoclast formation and bone resorption

*Selected by Welch *t* test with $P < .01$ differences.
†The ratio of the average expression of the SSPE pair to the normal pair was shown.

normal control group (*P* value .0006 by two-sided Student's *t* test). The relative expression levels of 519 and 520 were lower in stage III/IV SSPE patients than in stage I/II patients, but the differences were not significant (Figure 2).

An association study between −189T/G or 4214C/G polymorphisms of *GNLY* and SSPE showed no significant differences in the genotype and allele frequencies of the −189G/T or 4214 G/C polymorphisms between SSPE patients and controls, as shown in Table 2.

The two polymorphisms, −189G/T and 4214 G/C, were in perfect linkage disequilibrium, consisting of either the G-G or T-C haplotype.

Discussion

In the present study, we analyzed differences in the gene expression of PBMCs between SSPE patients and controls using a high-density oligonucleotide microarray containing approximately 30,000 human genes and found that *GNLY* had a significantly lower expression in SSPE patients. By quantitative RT-PCR, the relative expression levels of *GNLY* in PBMCs tended to decrease in 30 SSPE patients, whereas they significantly increased in 7 measles patients in comparison to those in 23 normal controls.

Granulysin is a recently defined cytolytic molecule released by cytotoxic lymphocytes (CTLs) and natural killer (NK) cells via granule-mediated exocytosis (Clayberger and Krensky, 2003; Lieberman, 2003), and its expression is at least in part dependent on inflammatory cytokines (Ma *et al*, 2002; Samten *et al*, 2003). Granulysin kills a variety of extracellular microorganisms, including bacteria, fungi, and

Table 2 Association study between *GNLY* −189G/T and 4214 G/C SNP, and SSPE

GNLY		*Normal controls*	*SSPE patients*	
−189G/T	*4214 G/C*	n*(%)*	n*(%)*	P *value*
		Genotype frequency		
GG	GG	27 (22)	6 (15)	NS
GT	GC	52 (42)	20 (50)	NS
TT	CC	45 (36)	14 (35)	NS
		Haplotype frequency		
G	G	106 (43)	32 (40)	NS
T	C	142 (57)	48 (60)	NS

Note. P values were calculated using the 2 × 2 or 2 × 3 chi-square test. NS: not significant.

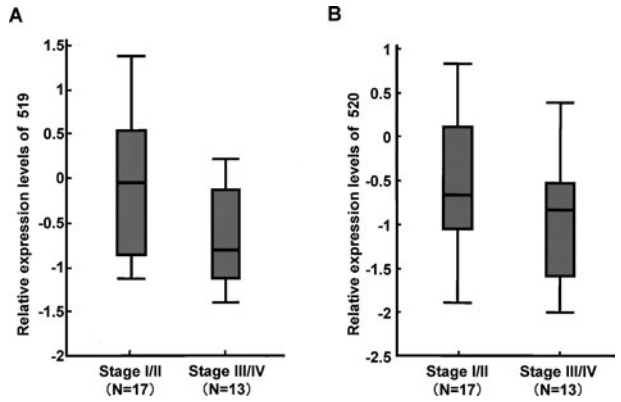

Figure 2 Expression levels of *GNLY* in PBMCs from stage I/II and III/IV SSPE patients. Box and whisker plots are shown in the same way as in Figure 1 for the (**A**) 519 and (**B**) 520 mRNA expressions in PBMCs from stage I/II and stage III/IV SSPE patients. The log-transformed values of the expression ratio to rRNA are shown. The values in parentheses indicate the number of samples. The log-transformed medians of the 519 mRNA expressions were −0.063 (−2.5 to 2.0) in stage I/II, and −0.80 (−2.4 to 0.57) in stage III/IV. The same medians of 520 mRNA expressions were −0.68 (−3.0 to 1.7) in stage I/II, and −0.74 (−2.4 to 0.49) in stage III/IV.

parasites, and, in combination with perforin, intracellular *Mycobacterium tuberculosis* by increasing the membrane permeability (Stenger *et al*, 1998; Kaspar *et al*, 2001; Anderson *et al*, 2003). It also induces the lysis of target cells, which is accompanied by the induction of cellular apoptosis. Although the role of granulysin in antiviral host-defense mechanisms is not well established compared to other organisms, the fact that virus-infected cells are eliminated by NK cells and CTLs mainly through exocytosis of cytolytic granules (e.g., perforin and granzymes) (Trapani *et al*, 1999) indicates the possibility that granulysin contained in cytolytic granules functions as an antiviral molecule. In support of this assumption, Ogawa *et al* (2003) recently reported the serum granulysin levels to be transiently elevated in patients with acute viral infections such as parvovirus B19 and Epstein-Barr virus (EBV). In addition, granulysin blocked the replication of a virus and triggered the apoptosis of infected cells *in vitro* (Hata *et al*, 2001). In the present study, we showed that *GNLY* was highly expressed in PBMCs from measles patients. MV infection causes transient, but profound lymphopenia with a reduction in the cell number of CD4+ T cells and CD8+ T cells (Arneborn *et al*, 1983). In addition, reduced NK activity was reported in the acute phase of measles (Griffin *et al*, 1990). Because MV-specific CD8+ CTLs play a pivotal role in eliminating MV-infected cells during acute measles (Griffin *et al*, 1994), the up-regulation of *GNLY* in PBMCs appears to reflect the remarkable activation of CD8+ CTLs as a compensatory mechanism for a reduced total number of CD8+ T cells and decreased NK activity during acute measles, thus suggesting that the release of granulysin through cytotoxic granules from CTL is involved in the host immune response against MV infection.

In contrast, the expression of *GNLY* in PBMCs tended to decrease in SSPE, especially in stage III/IV, although the difference was not statistically significant. Based on the present study showing a high expression of *GNLY* in acute measles, it is postulated that a low expression of *GNLY* suggestive of a reduced CTL response may thus be advantageous for establishing and maintaining a persistent MV infection. This finding might support a previous report that the CTL response to MV was reduced in SSPE patients (Dhib-Jalbut *et al*, 1989). The observation that inflammatory cytokines were not elevated in plasma of patients with SSPE (Tekgul *et al*, 1999) is also compatible with the low expression of *GNLY*. This postulated mechanism might be applicable to the development of CNS lesions in SSPE, because MV-infected neurons are targets of CTLs that enter the CNS from the peripheral blood (Ransohoff *et al*, 2003). However, because there were no differences in the distribution of *GNLY* SNPs between the patients and controls, we were not able to conclude that *GNLY* itself contributed to a genetic susceptibility to SSPE. It is possible that a certain *GNLY*-related gene that is directly responsible for a genetic susceptibility to SSPE played a role in the development of SSPE. Alternatively, it is possible that a low expression of *GNLY* in SSPE patients was not a cause but a result of a persistent viral infection. Low expression levels of *GNLY* in PBMCs were observed in patients with chronic active EBV and chronic hepatitis C infections (data not shown), a reduced expression of granulysin might be a secondary event in chronic viral infections.

In conclusion, granulysin might therefore play a role in the host defense against MV and it might also be involved in the pathogenesis or pathophysiology of SSPE. Further investigation should be needed to elucidate the role of granulysin in acute and chronic MV infections.

Methods

The patient population was composed of 40 Japanese patients with SSPE (27 males, 13 females, mean age ±SD; 12.4 ± 5.1 years), as described in our previous study (Kusuhara *et al*, 2000; Inoue *et al*, 2002; Torisu *et al*, 2004). All SSPE patients were diagnosed according to diagnostic criteria (Gascon, 1996), and classified by the Jabbour clinical staging method (Jabbour *et al*, 1969). For a microarray analysis, enough RNA samples were available from two SSPE patients in stage III (a 5-year-old girl and a 12-year-old boy) and two normal subjects (a 5-year-old girl and an adult man). For a quantitative RT-PCR, 30 SSPE patients (20 males, 10 females; 12.4 ± 5.1 years) whose cDNAs were available, 23 age-matched normal controls (12 males, 11 females, 15.5 ± 9.0 years), and 7 measles patients (5 boys, 2 girls, 3.1 ± 4.1 years) were studied. The SSPE patients were divided into two subgroups: stage I/II, $n = 17$ (12.3 ± 4.3 years) and stage III/IV,

$n = 13$ (12.7 ± 6.2 years). For SNP typing, DNA samples from all 40 patients were analyzed, and the normal control group comprised of 124 unrelated normal school children. Written informed consent was obtained from all the subjects and/or their parents. The Ethics Committee of Kyushu University, Japan approved the present study (No. 39).

PBMCs were separated immediately by density-gradient centrifugation using LSM (Cappel-ICN Immunobiologicals, Costa Mesa, CA) and frozen at −80°C. Total RNA was extracted from frozen PBMCs by an RNA extraction kit, ISOGEN (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. We also extracted total RNA from mixed buffy coats of two adults in the same way for the signal control. Total RNA was amplified using an Amino Allyl Message Amp aRNA kit (Ambion, Austin, TX). Briefly, double-stranded complementary DNA (cDNA) was synthesized from 2 μg of total RNA using oligo dT primer with a T7 RNA polymerase promoter site added to the 3′ end. After second-strand synthesis, *in vitro* transcription was performed in the presence of amino allyl UTP to produce multiple copies of amino allyl labeled complementary RNA (cRNA). Amino allyl labeled cRNA were purified, and then reacted with *N*-hydroxysuccinimide esters of Cy3 (Amersham Pharmacia Biotech, Piscataway, NJ) for samples, Cy5 (Amersham Pharmacia Biotech) for buffy coats following the manufacturer's instructions. Dye molecules were separated from labeled products using Micro Bio-Spin P-30 tris chromatography columns (Bio-Rad, Hercales, CA). Cy3-labeled cRNA were mixed with the same amount of Cy5-labeled cRNA. Before hybridization, labeled cRNAs were fragmented by incubation at 94°C for 15 min in fragmentation buffer (40 mM Tris-acetate, pH 8.1, 100 mM potassium acetate, 30 mM magnesium acetate tetrahydrate). Fragmented cRNAs were added to hybridization buffer (5× SSC [1 × SSC solution contains 0.15 M sodium chloride and 0.015 M sodium citrate], 0.5% sodium dodecyl sulfate [SDS], 4× Denhardt's solution, 100 μg/ml salmon sperm DNA, 10% formamide). Hybridization was performed for 16 to 20 hours by incubating 48 μl of samples to each Acegene Human oligo chip 30 K slide (Hitachi Software Engineering, Yokohama, Japan), with a total of three slides, and the gene expression data for approximately 30,000 human genes was evaluated. Each slide was rinsed with the recommended buffer, and then dried and fluorescence signals were detected using a fluorescent image analyzer FLA-8000 (Fuji Film, Tokyo, Japan). Separate images were acquired for Cy3 and Cy5. When the analysis was carried out, the mRNA signals of the PBMCs from each individual subject were compared with those from mixed buffy coats.

The data were analyzed using Arrayvision version 6.0 (Imaging Research, Ontario, Canada), and Genespring version 5.0 (Silicon genetics, Redwood City, CA). The digital images were aligned to a template provided by the manufacturer and the hybridization intensities for each spot were measured using Arrayvision. The signal and background pixel classifications were determined by manually positioning the grid of the circles over the array image. The signal and background intensities were determined based on the median pixel values. The local background values were determined as the average of four background spots around each gene spot. Net signal was determined by the subtraction of the local background. Next, the signal data were changed into text for further processing. According to the Genespring instructions, these data then underwent per spot intensity dependent Lowess normalization (Yang *et al*, 2002) to correct any dye and/or slide bias. The genes, of which absolute signal values in the Cy5 or Cy3 channels were less than twice that of the backgrounds, or in which the standard deviations of the normalized expression levels were higher than 0.5 in either the patients or controls, were excluded. A total of 12,121 genes among the 30,000 genes were considered to show a reliable expression and thus were used for the subsequent analysis.

cDNA syntheses were performed as described previously (Ihara *et al*, 2000). cDNA generated from phytohemagglutinin (PHA)-stimulated PBMCs was used to obtain a standard curve. After completing the reverse transcriptase reaction, samples were diluted 1/10 with sterile water, and 0.5 μl of the diluted cDNA was used for PCR amplification in a 25 μl reaction mixture. *GNLY* is alternatively spliced, resulting in two transcripts, 519 (NM_012483) and 520 (NM_006433). The oligonucleotide sequence of *GNLY* mounted on the Human oligo chip 30 K slide is the common part of both transcripts. The 519 transcript includes an additional 242-bp segment within intron 1 of the *GNLY*. The inclusion of this additional segment results in the utilization of a different translation start codon, but both isoforms produce the same 9-kDa mature form via post-translational processing (Yabe *et al*, 1990). 519 is mainly expressed in T cells, and 520 is mainly expressed in NK cells. Confirmatory quantitative RT-PCRs for mRNAs of *granulysin* isoforms 519 and 520, and internal control ribosomal RNA (rRNA) were performed using an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol as described previously (Ihara *et al*, 2000). The sequences of the intron-spanning amplification primers and TaqMan probe for granulysin isoform 519 were as follows: GGATGGAAGGTCTGGTCTTCT, AACAGGTCACCCTGGGGG, and FAM-AGCCCACCTGCGTGATGGG-TAMRA. Those for granulysin isoform 520 were TCCTTGCAGCCATGCTCC, AACAGGTCACCCTGGGGG, and FAM- CTGCCCGTGCCTGGCCCA-TAMRA. For quantitative RT-PCR of rRNA, we used predeveloped TaqMan ribosomal control reagent (P/N4308329; Applied Biosystems). The crude expression level of each target gene was divided by that of an internal control,

rRNA, and then the final expression level was described as a ratio to that of PHA-stimulated PBMCs. Each run was performed in duplicate. The variation in the final expression levels in each duplicated samples was mostly less than 20%, and the mean expression level of duplicates was used for further calculations.

GNLY consists of five exons extending 4.2 kb in chromosome 2. Up to now 25 single nucleotide polymorphisms (SNPs) have been reported from nucleotide position −1200 bp to the 3′ end of *GNLY,* and the frequencies of these SNPs in the Japanese population were reported (Hirakawa *et al*, 2002). Among them, we analyzed −189 T/G and 4214C/G polymorphisms in 40 unrelated SSPE patients and 124 unrelated normal school children. Genomic DNA was extracted from their PBMCs using QIAamp DNA Blood Kit (QIAGEN, Tokyo, Japan). We used TaqMan Assays-on-Demand SNP Genotyping Products for analysis of −189T/G and 4214C/G polymorphisms (Assay ID: C_3227280_1 and C_3227296_10).

Before comparing the basal expression levels of mRNA in various groups, we examined the normality of their distributions. The mRNA levels of both granulysin isoforms deviated significantly from Gauss distribution ($P < .001$), and the extent of this deviation was substantially decreased after a log transformation. We therefore used the natural logarithms of the mRNA levels as the dependent variable in the following analysis to identify any differences among these groups. However, because the levels of 519 mRNA in the normal group did not fit Gauss distribution even after log-transformation ($\chi^2 = 3.91 > \chi^2_{(0.95)} = 3.84$), we performed a nonparametric analysis (Kruskal-Wallis H test followed by Mann-Whitney U test) of the log-transformed levels of 519 mRNA. The log-transformed levels of all other groups fitted the Gauss distribution. We used one-way analysis of variance (ANOVA) followed by the two-sided t test to evaluate the log-transformed levels of 520 mRNA. We used Welch's t test to compare the normal and SSPE groups, because the variances of these groups were unequal. We used Student's t test to compare the normal and measles groups. To compare stages I/II and III/IV of SSPE, we used Welch's t test. A P value of $< .05$ was considered to be significant. However, when comparing the normal and SSPE groups and the normal and measles groups, the statistical significance level was specified using the Bonferroni correction (the number of analyses was 2), and a P value of $<.025$ was considered to be significant.

In the SNP analysis, the differences in the allele or genotype frequencies between two groups were evaluated by a chi-square analysis with a 2×2 contingency table.

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