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Short Communication

Analysis of gene-expression profiles by oligonucleotide microarray in children with influenza

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In order to clarify the mechanism of the host response to influenza virus, gene-expression profiles of peripheral blood obtained from paediatric patients with influenza were investigated by oligonucleotide microarray. In the acute phase of influenza, 200 genes were upregulated and 20 genes were downregulated compared with their expression in the convalescent phase. Interferon-regulated genes, such as interferon-induced protein with tetratricopeptide repeats 2 (IFIT2) and vipirin, were strongly upregulated in the acute phase. Gene ontology analysis showed that immune response genes were highly overrepresented among the upregulated genes. Gene-expression profiles of influenza patients with and without febrile convulsion were also studied. In patients with febrile convulsion, 22 genes were upregulated and five were downregulated compared with their expression in patients without febrile convulsion. These results should help to clarify the pathogenesis of influenza and its neurological complications.

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Influenza virus is one of the major pathogenic organisms in human populations, and it produces a spectrum of clinical responses ranging from upper respiratory illness to central nervous system involvement (Delorme & Middleton, 1979; Wright & Webster, 2001). The host response to influenza virus has not been fully elucidated in humans. Influenza virus replicates in the epithelial cells of the upper respiratory tract, which has several mechanisms to protect against influenza infection. Patients with influenza have respiratory symptoms, such as cough and rhinorrhoea, owing to mucosal inflammation and an influx of polymorphonuclear cells (Wright & Webster, 2001). In addition to these symptoms, most patients with influenza suffer from systemic symptoms, including high fever, myalgias and malaise. To date, the initial responses to influenza virus infection have been investigated mainly in the respiratory tract (Hayden *et al.*, 1998; Kaiser *et al.*, 2001; Skoner *et al.*, 1999); however, the global host response to the virus has not been investigated in humans.

Recently, there has been an increase in the number of reports of influenza-associated encephalopathy in Japan and other countries (Kasai *et al.*, 2000; Morishima *et al.*, 2002; Newland *et al.*, 2003). Patients with influenza-associated encephalopathy often develop multi-organ failure and have high rates of morbidity and mortality. In addition to encephalopathy, influenza virus infection is associated with a higher incidence of febrile convulsions compared with other viral infections (Chiu *et al.*, 2001; Kawada *et al.*, 2004). The pathogenesis of these complications has not been fully understood, although it may be that encephalopathy and febrile convulsion are provoked by the same mechanism (Kawada *et al.*, 2003b).

The aim of the present study was to analyse gene-expression profiles in the peripheral blood of influenza virus-infected patients in order to reveal the mechanisms of the host response to influenza virus. An oligonucleotide microarray was used to reveal global host responses to influenza virus

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Details of genes up- and downregulated during the acute phase of infection are available as supplementary material in JGV Online.

infection in children. This technique allows the expression patterns of thousands of genes to be studied in parallel. In addition, the gene-expression profiles of influenza patients with or without febrile convulsion were compared.

Nine patients with influenza who presented during one of two influenza seasons (2002–2004 seasons) were enrolled in this study. Their ages ranged from 3 months to 4 years (median 2 years) and all had typical clinical signs and symptoms of influenza, such as acute onset of fever, cough, chills and nasal congestion. Five patients with febrile convulsion were included. They had convulsions of short duration (1–5 min) provoked by fever but recovered without any sequelae. Four patients without febrile convulsion from the same district as those with febrile convulsions were selected as age-matched controls. None of the patients had underlying disease. Cultures of blood, cerebrospinal fluid and throat swabs showed no indication of central nervous system infection by other viruses or bacteria. They also did not receive aspirin or folk remedies during the influenza episodes. Influenza infection was confirmed by a fourfold or greater increase in haemagglutinin inhibition test titres and/or virus antigen detection in the throat using the latex agglutination test. All viruses were identified as type A influenza (H3N2). At the time of enrolment, antibody titres against H3N2 were negative (<10) in six of seven tested patients, suggesting that most patients were naive to influenza virus. Only one patient had received influenza vaccination before infection. As controls, four healthy children were also enrolled in this study. All samples were obtained from Aichi prefecture, which is located in the centre of Japan. Informed consent was obtained from the parents of all children participating in this study. This study was approved by the ethical committee of Nagoya University Graduate School of Medicine.

Peripheral blood was collected from patients both in the acute phase of the disease [0–2 days after the patients had high-grade fever ($>38{\cdot}0$ °C)] and in the early convalescent phase (1–5 days after body temperature returned to normal). Whole blood (2·5 ml) was drawn directly into a PAX gene blood RNA tube (Qiagen), and total RNA was purified with PAX gene blood RNA kits (Qiagen) according to the manufacturer's protocols. White blood cells in the acute phase of influenza contained a mean of 61 % granulocytes, 31 % lymphocytes and 5 % monocytes. Total RNA (400 ng) was amplified and labelled using the Agilent Low RNA Input fluorescent linear amplification kit (Agilent Technologies). For each patient, acute-phase and early convalescent-phase samples were labelled with Cy3 and Cy5, respectively. Hybridizations on the microarray slide of Human 1A (Agilent Technologies), which contains 17 086 sequenced human genes, were carried out for 17 h in a rotating hybridization oven using an *in situ* hybridization kit (Agilent Technologies). The slides were washed as indicated in the protocol and were then scanned with an Agilent Scanner (G2565).

Microarray expression data were obtained using the Feature Extraction software (Agilent Technologies). The raw data on the intensity of gene expression in the acute phase were normalized by Z transformation to obtain Z scores (Cheadle *et al.*, 2003). The latter were calculated by subtracting the mean intensity of gene expression in the convalescent phase from the mean intensity of gene expression during the acute phase for each gene and then dividing that result by the standard deviation of gene expression intensity during the convalescent phase. Using this approach, it was possible to compare the results from different experiments. A gene was defined as being upregulated in the acute phase if the Z score was greater than 1·96 ($P<0{\cdot}05$) and downregulated if the Z score was less than $-1{\cdot}96$. In addition, levels of gene expression were compared among clinical categories and Z scores were calculated for each clinical category [no febrile convulsion, Z (N); febrile convulsion, Z (FC)]. Differences in gene expression were evaluated by comparing these scores. The transcription of a gene was considered to be upregulated more in patients with febrile convulsion than in patients with no febrile convulsion if $Z(\mathrm{FC})-Z(\mathrm{N})$ was greater than 3·92 ($P<0{\cdot}05$). The applicability of these methods to microarray data was examined and verified in previous studies (Hong *et al.*, 2004; Kim *et al.*, 2003).

The molecular changes associated with influenza virus infection *in vivo* were investigated by microarray analysis of RNA from peripheral whole-blood samples using a 17 086-element oligonucleotide microarray. Gene-expression intensity data collected from all nine patients with influenza infection were used. Overall, 200 genes were found to be upregulated ($Z>1{\cdot}96$) and 20 genes were found to be downregulated ($Z<-1{\cdot}96$) in acute-phase influenza. Fig. 1 shows the Z scores of 15 genes that were upregulated and 15 genes that were downregulated. Interferon-induced protein with tetratricopeptide repeats 2 (IFIT2) and vipirin (cig5), which is also induced by interferon, were respectively the most and second most upregulated genes in acute-phase infection. Protein tyrosine phosphatase receptor type C (PTPRC), which was the third most upregulated gene, is an essential regulator of T- and B-cell antigen receptor signalling. Prolactin (PRL), which is a growth hormone that stimulates lactation, was the most downregulated gene in acute-phase influenza. Summaries of the genes that were up- or downregulated in the acute phase are shown in Supplementary Table S1 available in JGV Online. RNA from healthy children was also analysed using the same assay system. Of 200 genes that were upregulated in the acute phase of influenza, only three were upregulated ($Z>1{\cdot}96$) in the control children (Supplementary Table S1). Similarly, of 20 genes that were downregulated in acute influenza, only two genes were downregulated (Supplementary Table S1). Thus, in the group of healthy children, only a few genes were up- or downregulated, indicating that the up- or downregulation seen in influenza patients is specific to influenza infection.

To determine the categories of significantly overexpressed genes, Expression Analysis Systematic Explorer (EASE) software version 2.1 (National Institutes of Health, USA) was used (Hosack *et al.*, 2003). Each gene was classified

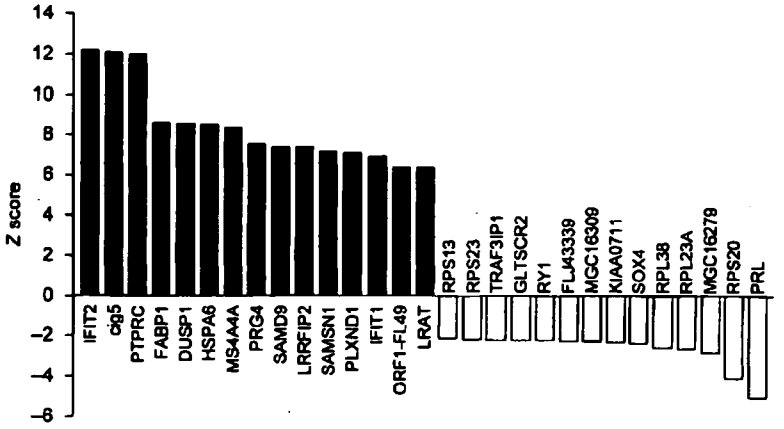

Fig. 1. Genes that were upregulated (filled bars) or downregulated (open bars) in patients with acute-phase influenza virus infection. The transcription level of each gene is expressed as the *Z* score, which was calculated as: *Z* (gene 1) = [mean intensity of gene 1 (acute phase) − mean intensity of gene 1 (convalescent phase)]/ standard deviation of intensity of gene 1 (convalescent phase).

according to its ontology, by which genes are organized into hierarchical categories based on biological process, molecular function and cellular component (Ashburner *et al.*, 2000; Boyle *et al.*, 2004). Based on an analysis using EASE software, several gene ontology categories were identified as being overrepresented (EASE score $<0{\cdot}05$). Genes assigned to the category of immune response genes were the most overrepresented among the upregulated genes (EASE score 0·00000001). Genes in ontology categories associated with the immune response, such as defence response and response to biotic stimulus, were also overrepresented (EASE scores of 0·00004 and 0·00007, respectively). Representative gene ontology categories considered to be overrepresented are shown in Fig. 2.

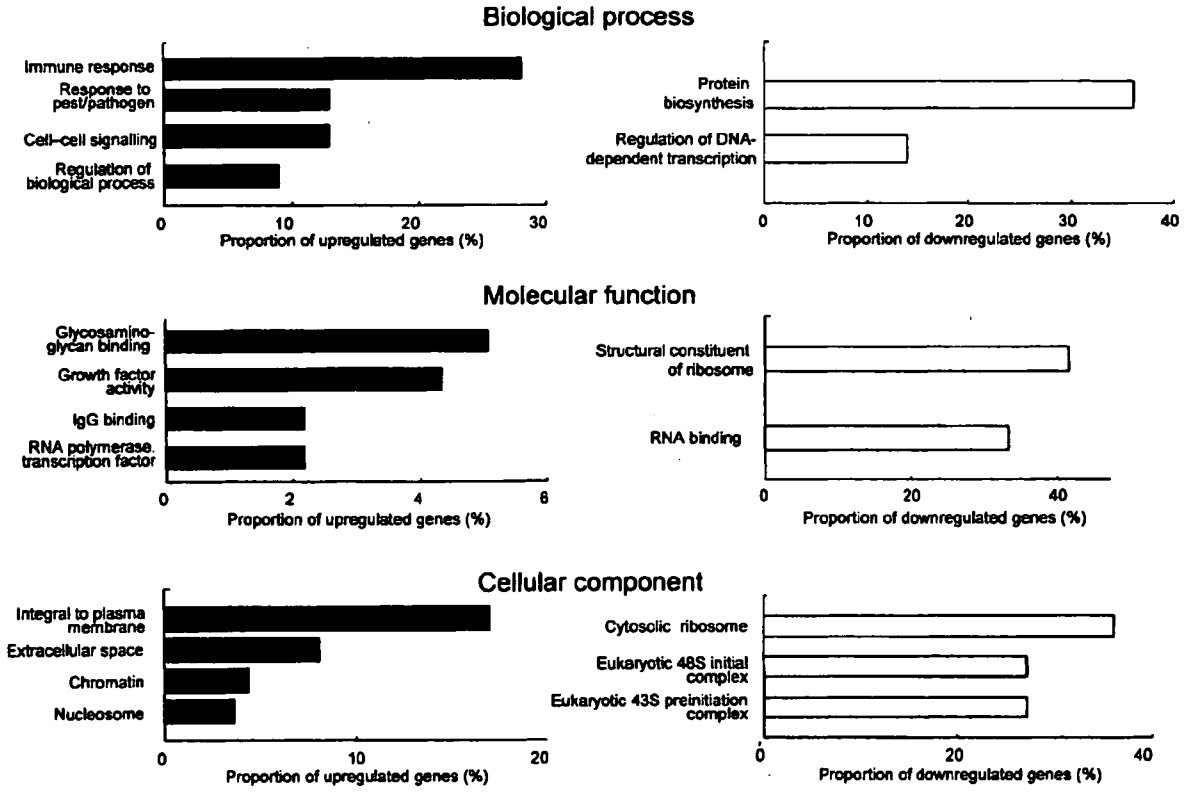

Fig. 2. Percentages of up- and downregulated genes according to gene ontology categories. Upregulated (200) and downregulated (20) genes in acute-phase influenza virus infection were categorized by gene ontology analysis using EASE software version 2.1.

Table 1. Up- and downregulated genes in patients with febrile convulsion

Z scores were calculated as described in the text. Significance thresholds were set at >3·92 or <−3·92.

Z (FC) − *Z* (N)	GenBank accession no.	Gene symbol	Gene name	Gene ontology (biological process)
Upregulated genes				
6·50	NM_145344	APOL1	Apolipoprotein L	Lipid metabolism
5·53	NM_057089	AP1S1	Adaptor protein complex 1 sigma 1 subunit	Endocytosis; intracellular protein transport
5·39	NM_019858	GRCA	Gene-rich cluster, A gene	G-protein-coupled receptor protein signalling pathway
5·32	AB065742	OR5M1	Olfactory receptor, family 5, subfamily M, member 1	Signal transduction
5·21	NM_006309	LRRFIP2	Leucine-rich repeat- (in FLII) interacting protein 2	Unclassified
5·13	NM_013956	NRG1	Neuregulin 1	Cell differentiation; embryonic development; neurogenesis
4·84	NM_018991	DKFZP434A0131	DKFZp434A0131 protein	Unclassified
4·69	NM_020632	ATP6N1B	ATPase (H^+ transporting) lysosomal V0 subunit A isoform 4	Proton transport; regulation of pH
4·50	NM_006943	SOX22	SRY (sex-determining region Y)-box 22	Regulation of transcription from Pol II promoter
4·48	NM_014746	RNF144	Ring-finger protein 144	Protein ubiquitination
4·42	AY166852	RAB41	RAB41, member RAS oncogene family	Intracellular protein transport; small GTPase-mediated signal transduction
4·42	NM_052954	CYYR1	Cysteine- and tyrosine-rich 1	Biological process unknown
4·36	NM_021615	CHST6	Carbohydrate sulfotransferase 6	*N*-Acetylglucosamine metabolism
4·35	NM_004570	PIK3C2G	Class II phosphoinositide-3-kinase gamma	Intracellular signalling cascade
4·32	NM_005164	ABCD2	ATP-binding cassette, subfamily D (ALD), member 2	Fatty acid metabolism; transport
4·23	NM_033386	MIRAB13	Molecule interacting with Rab13	Vesicle-mediated transport
4·19	NM_033506	FBXO24	Protein containing an F-box domain	Protein ubiquitination
4·09	NM_003214	TEAD3	TEA domain family member 3	Regulation of transcription, DNA-dependent
4·01	NM_025216	WNT10A	Wingless-type MMTV integration site family member 10a	Development; frizzled-2 signalling pathway
4·01	NM_025152	NUBPL	Nucleotide-binding protein-like	Unclassified
4·00	NM_031921	ATAD3B	ATPase family, AAA domain-containing 3B	Cell division
3·94	NM_080923	PTPRC	Protein tyrosine phosphatase, receptor type, C	Cell-surface receptor-linked signal transduction; protein amino acid dephosphorylation
Downregulated genes				
−9·24	NM_018437	HEMGN	Haemogen	Signal transduction
−7·50	NM_024021	MS4A4A	Membrane-spanning 4-domain subfamily A member 4A	Signal transduction

Table 1. cont.

Z (FC) − Z (N)	GenBank accession no.	Gene symbol	Gene name	Gene ontology (biological process)
−5·80	NM_013363	PCOLCE2	Procollagen C-endopeptidase enhancer 2	Transport
−4·64	NM_018571	ALS2CR2	Amyotrophic lateral sclerosis 2 chromosome region candidate 2	Protein amino acid phosphorylation
−4·56	NM_001069	TUBB	Tubulin, beta polypeptide	Microtubule polymerization; microtubule-based movement; natural killer cell-mediated cytolysis

Differences in gene expression among clinical categories were investigated by comparing the Z scores. Compared with gene expression in patients without febrile convulsions, 22 genes were found to be upregulated in patients with febrile convulsion [Z (FC) − Z (N) >3·92]. A summary of these 22 genes is given in Table 1. By contrast, five genes were found to be downregulated in patients with febrile convulsion [Z (FC) − Z (N) < −3·92]; these are also listed in Table 1. Gene ontology analysis was also carried out for upregulated or downregulated genes in patients with febrile convulsion, but no gene ontology categories were found to be significantly overrepresented (EASE score >0·05).

In this study, an oligonucleotide microarray was used to reveal global host responses to influenza A virus infection. Microarray technology has been used previously to investigate the pathogenesis of this disease. Gene-expression profiles of lung cells and middle-ear epithelial cells infected with influenza virus *in vitro* have been examined (Geiss *et al.*, 2002; Tong *et al.*, 2004). Furthermore, the host immune response to influenza A virus was investigated using the lungs of influenza-infected mice and pigtailed macaques (Baskin *et al.*, 2004; Kash *et al.*, 2004). These *in vitro* and animal studies showed that influenza virus infection results in a significant induction of genes involved in the interferon pathway. To investigate the global host response to influenza, gene-expression profiling of peripheral blood from patients with influenza was carried out using a microarray. We showed that interferon-regulated genes, such as IFIT1, IFIT2 and vipirin, were strongly upregulated in the acute phase of influenza. Furthermore, many other genes associated with the immune response were upregulated. These results are consistent with the gene-expression profiles obtained by microarray analysis of influenza virus-infected lung cells (Geiss *et al.*, 2002; Tong *et al.*, 2004). It has been shown that interferon-regulated genes are activated *in vitro* in human peripheral blood infected with influenza virus (Ronni *et al.*, 1995). Our results are compatible with these gene-expression profiles, indicating that our method is a reliable approach to investigate the pathogenesis of influenza. The global host response to influenza virus could be established in clinical samples.

The clinical manifestations of influenza in children are similar to those seen in adults, but there are also some distinct differences between these two populations. In children, influenza is accompanied occasionally by febrile convulsion and rarely by encephalopathy (Chiu *et al.*, 2001; Morishima *et al.*, 2002). Influenza-associated encephalopathy is a severe disease with high mortality. Febrile convulsion, which results in impaired consciousness lasting <24 h, may be difficult to differentiate from encephalopathy, and it may be that encephalopathy and febrile convulsion are provoked by the same mechanisms (Kawada *et al.*, 2003b). In this study, 22 genes were upregulated and five genes were downregulated in patients with febrile convulsion compared with their expression in patients without febrile convulsion. Although gene ontology analysis did not identify significantly overrepresented categories for these genes, we found that some interesting genes were included. Apolipoprotein L (APOL1) and ATP-binding cassette, subfamily D, member 2 (ABCD2) are associated with fatty acid metabolism (Duchateau *et al.*, 1997; Holzinger *et al.*, 1999). This finding is of interest because Reye's syndrome, which involves acute encephalopathy, is associated with a disturbance in fatty acid metabolism (Ogburn *et al.*, 1982) and often follows influenza virus infection and salicylate therapy in children (Belay *et al.*, 1999; Starko *et al.*, 1980). None of the patients enrolled in this study received salicylate, but it may be that patients with genetic abnormalities in fatty acid metabolism are more likely to experience neurological complications. ABCD2 is also an adrenoleukodystrophy-related protein. Adrenoleukodystrophy is characterized by demyelination in the central nervous system (Moser, 2000). Furthermore, neureglin 1 (NRG1), which induces the growth and differentiation of neuronal and glial cells (Law *et al.*, 2004), was also upregulated.

The pathogenesis of influenza-associated encephalopathy remains unclear. Viral RNA is rarely detected in the cerebrospinal fluid, and viral antigen is not present in the brain (Ito *et al.*, 1999; Kawada *et al.*, 2003a). Pathological findings, including the lack of detectable viral antigen and inflammatory cells in brain tissues, suggest that direct viral

invasion and inflammation are therefore unlikely to be the cause of this disease. Instead, several studies have revealed that serum concentrations of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and IL-1β, are elevated and are related to the clinical severity of the encephalopathy (Aiba *et al.*, 2001; Ito *et al.*, 1999). In a previous study, we showed that transcription of IL-6 and IL-10 genes in peripheral leukocytes is upregulated in patients with influenza-associated encephalopathy (Kawada *et al.*, 2003b). Furthermore, several studies have suggested an association between febrile convulsion and pro-inflammatory cytokines (Straussberg *et al.*, 2001; Virta *et al.*, 2002). In this study, the transcription levels of pro-inflammatory cytokine genes in patients with febrile convulsion were not significantly different from those in patients without febrile convulsion. Several reasons may account for this discrepancy. Firstly, unlike in patients with influenza-associated encephalopathy, the transcription levels of pro-inflammatory cytokines may not be upregulated in patients with febrile convulsion. Febrile convulsions might not be an appropriate marker of neurological complications and our results may not be generalized to other forms of neurological complications, such as Reye's syndrome, acute necrotizing encephalopathy and myelitis. Secondly, it may be that the differences in the transcription levels were too small to be detected by microarray techniques.

To our knowledge, this is the first study to show the global host responses in peripheral blood of patients with influenza. The data suggest that microarrays can be applied to the investigation of clinical samples from patients with acute viral infection. Our results should help to clarify the pathogenesis of influenza and its neurological complications, including encephalopathy.

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PostScript

LETTERS

Paraneoplastic cerebellar degeneration in olfactory neuroepithelioma

Anti-Hu antibody was first discovered in patients with paraneoplastic encephalomyelitis associated with small cell lung cancer (SCLC). This antibody recognises proteins comprised in the Hu family expressed by neuronal cells as well as SCLC. After the first report, anti-Hu antibody was found in other neoplasms including prostate and breast cancer, adrenal carcinoma, chondromyxosarcoma, neuroblastoma, and neuroendocrine neoplasms at other sites.[1] Olfactory neuroepithelioma (9523/3)[2] is thought to differ from classic neuroblastoma (9500/3) in its expression pattern of tyrosine hydroxylase, *MYCN* amplification, and fusion of the Ewing sarcoma gene and the Friend leukaemia virus integration 1 gene or the ETS related gene.[3]

Anti-Hu antibody in association with olfactory neuroepithelioma has not been reported previously. We report a patient with cerebellar ataxia that paralleled the recurrence of the tumour. Serum and cerebrospinal fluid (CSF) from the patient contained anti-Hu antibody, and the olfactory neuroepithelioma resected from the patient expressed Hu antigen.

CASE REPORT

Seven years before admission, a 65 year old man presented with olfactory neuroepithelioma that had invaded the orbit and frontal lobe. The tumour was dissected surgically, and dura mater graft was not used in the surgery. The patient underwent irradiation (total dose of 50 Gy). The tumour recurred at the parotid gland in January 2001, and there was gait instability. The patient consulted a neurologist, but there was no specific finding. The recurrent tumour was surgically dissected; however, the instability progressed rapidly, and at the patient's admission in November 2001, he needed support when walking. There was neither alcoholism nor family history of cerebellar ataxia. His parents were not consanguineous.

General physical examination was negative. There was no lymphoadenopathy. He was alert and mentally normal. Olfactory sensation had been decreased since the first surgery, there was a downbeat nystagmus, and muscle strength was maximum. Both superficial and deep sensation were normal. Deep tendon reflex was symmetrical and normal, Romberg test was negative, and no pathological reflex was found. Nose–finger–nose test was normal, but heel–shin test was poor. Dysmetria was marked in both legs. His gait was wide based and ataxic, and tandem gait was impossible. There was no dysarthria.

Haematological studies, blood chemical analyses, and serological studies were normal. Tumour markers including α-fetoprotein, prostate specific antigen, pro-gastrin releasing peptide, neurone specific enolase, sialyl Lewis (a) (CA19–9), and sialyl Lewis (x) (SLX) were within normal limits. Levels of vitamin B1 and B12 were normal. Protein level in cerebrospinal fluid (CSF) was increased to 105 mg/dl with normal cellularity. Myelin basic protein and oligoclonal IgG band was negative. IgG index was 0.6. No malignant cells were found in the CSF. Nerve conduction study was normal. Short sensory evoked potentials of upper and lower limbs were normal. Electroencephalogram showed beta rhythm at the bilateral frontal region, with otherwise normal findings.

Computed tomography (CT) showed no lung tumour. Magnetic resonance imaging (MRI) showed bilateral leukoaraiosis at bilateral frontal lobes that had been present since after the first surgery. The cerebellum was slightly atrophic.

Titres of anti-Hu antibody in the serum and CSF were 1:1920 and 1:64, respectively (indirect immunofluorescence and Western blotting for recombinant HuD). Serum:CSF antibody titre ratio was 30. The ratio for (CSF/serum antibody titre)/(CSF/serum albumin) was 1.8. These values indicated that intrathecal synthesis of anti-Hu antibody had stopped at this time point. Other antineuronal antibodies including anti-Yo, Ri, CV2, Tr, Ma, amphiphysin, and glutamic acid decarboxylase were all negative. Systemic examination including ^{67}Ga-citrate scintigraphy did not disclose malignant tumours. Immunohistochemistry with anti-HuD antibody (Santa Cruz, sc-5977, ×100) revealed that a part of the tumour expressed Hu protein (fig 1).

Over the course of 4 years after discharge, the cerebellar ataxia did not worsen further in the absence of immunological treatment. Follow up thoracic CT and tumour marker study did not disclose other malignant tumours. There was no evidence of the recurrence of olfactory neuroepithelioma.

CONCLUSION

This patient presented cerebellar ataxia of the trunk and lower limbs that progressed rapidly within approximately 6 months after the second surgery and stabilised thereafter.

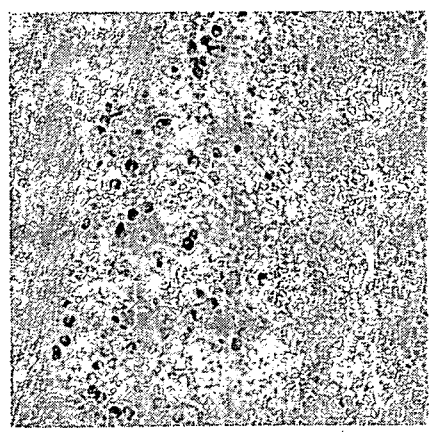

Figure 1 Immunohistochemistry using anti-HuD antibody. A part of the patient's tumour expressed HuD antigen (×400).

This clinical course is not inconsistent with the natural course of paraneoplastic cerebellar degeneration. Although isolated cerebellar ataxia in anti-Hu antibody positive patients is rare (4/200 patients),[1] a high titre of serum anti-Hu antibody (1:1920) corroborated the diagnosis of paraneoplastic syndrome.[1] The expression of the HuD protein by the olfactory neuroepithelioma confirmed the diagnosis.

Olfactory neuroepithelioma is a neuroectodermal neoplasm that arises from the olfactory epithelium. It is distinguished from classic neuroblastoma as described by Sorensen *et al*.[3] Unlike neuroblastoma, olfactory neuroepithelioma shows differentiation to the neural processes and glandular structure and is rarely associated with catecholamine secretion. In addition, olfactory neuroepithelioma expresses epithelial markers such as cytokeratin and a 34 kDa epithelial membrane glycoprotein recognised by monoclonal antibody named Ber-EP4. The tumour in this case expressed both Ber-EP4 and cytokeratin (see Okabe *et al*[4], case no. 6). Moreover, it also expressed luteinising hormone releasing hormone. The expression pattern of Ber-EP4 and cytokeratin was heterogeneous in this tumour.[4] These findings suggest that the tumour in this case had arisen from the olfactory placode and was distinct from classic neuroblastoma arising from the neural crest.[5] This neuroepithelial tumour has not been reported to be associated with paraneoplastic syndrome. Our data clearly demonstrate the expression of Hu antigen by the olfactory neuroepithelioma cells and the presence of Hu antibody in his serum and CSF. It is interesting that neurological manifestations developed in parallel with the recurrence of the tumour. The recurrence might have enhanced immune response. Despite resection of the recurrent tumour, the cerebellar ataxia worsened for several months after surgery. However, it did not progress thereafter. In patients with neurological symptoms and Hu antibody, olfactory neuroepithelioma should be considered when a neoplasm is not found at the common sites such as the lung or breast.

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Acute limbic encephalitis: A new entity?

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Abstract

Clinical cases similar to herpes simplex virus (HSV) encephalitis have accumulated in Japan. Detailed examinations have failed to demonstrate HSV infection. Recently, these cases have been named "non-herpetic acute limbic encephalitis". Only a single autopsy case was so far reported in an abstract form, because many cases showed a good prognosis. The case presented here was that following fever, a 59-year-old woman developed disturbance of consciousness and uncontrollable generalized seizures. Brain MRI revealed abnormal signals in the bilateral medial temporal lobe and along the lateral part of the putamen. Autoantibody against the NMDA glutamate receptor (GluR) IgM-ε2 was detected in the serum, and the GluR IgG-δ2 antibody was positive in cerebrospinal fluid. She died 12 days after onset. An autopsy examination revealed scattered foci consisting of neuronal loss, neuronophagia and some perivascular lymphocytic infiltration in the hippocampus and amygdala, but no haemorrhagic necrosis in the brain. HSV-1, -2 and human herpes virus-6 were negative immunohistochemically. We believe that our autopsy case may contribute to understanding the neuropathological background of non-herpetic acute limbic encephalitis.

Keywords: Acute encephalitis; Status epilepticus; Autopsy; Non-herpetic acute limbic encephalitis; Herpes simplex encephalitis

Limbic encephalitis is usually considered to be paraneoplastic, occurring subacutely in association with specific neuronal antibodies [2]. Among the cases with reversible acute or subacute non-paraneoplastic limbic encephalitis, voltage-gated potassium channel (VGKC) antibodies have been reported [12]. Autoantibodies against the NMDA glutamate receptor (GluR), which is considered to be related causally to partial seizures [11], were detected in the acute non-herpetic encephalitis [3].

In Japan, acute encephalitis, in which the clinical picture was comparable with that of herpes simplex virus (HSV) encephalitis but where evidence of HSV infection was not demonstrated, has been reported [5]. Recently, these cases have been named "non-herpetic acute limbic encephalitis" as a possible new subgroup of limbic encephalitis [5,9]. It has been proposed that mild infections and immunological process are the cause of this disease from clinical findings and cerebrospinal fluid (CSF) cytokine levels, elevated level of interleukin-6 [5,9] and unelevated level of interferon-γ [1]. Moreover, it has been indicated that acute limbic encephalitis, HSV encephalitis and other acute limbic encephalitis were etiologically interrelated, because cases of limbic encephalitis similar to non-herpetic acute limbic encephalitis were reported [1,9].

Many previously reported cases of non-herpetic acute limbic encephalitis have shown a rather favorable prognosis [1,4,5,7,8,10]. For this reason, only a single autopsy case was so far reported in an abstract form [7]. We believe that this report contributes to understanding the neuropathological background of the acute limbic encephalitis of unknown etiology.

One week after a fever, a 59-year-old woman developed progressive disturbance of consciousness following generalized tonic seizures. The brain computed tomography showed no abnormalities. CSF examinations showed mononuclear cells 10 μl/l, protein 50 mg/dl and glucose 143 mg/dl. The seizures continued, even though multiple anticonvulsants were administered and mechanical ventilation was performed. Eight days after the onset of unconsciousness and seizures, brain magnetic resonance imaging (MRI) with T2-weighted and FLAIR images revealed high signal intensities in the bilateral medial temporal lobes and along the lateral part of the putamen (Fig. 1). She was admitted to our hospital 10 days after the onset of the seizures. She showed marked emaciation and pneumonia complications. Recurrence of generalized tonic seizures

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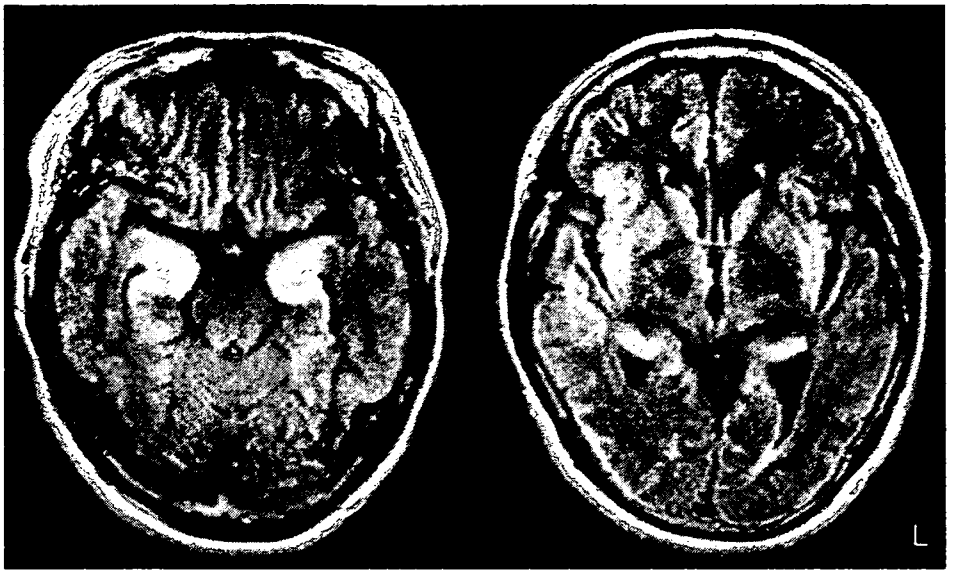

Fig. 1. FLAIR MRI images. High signal intensity is seen in the bilateral medial temporal lobe and the lateral part of the putamen.

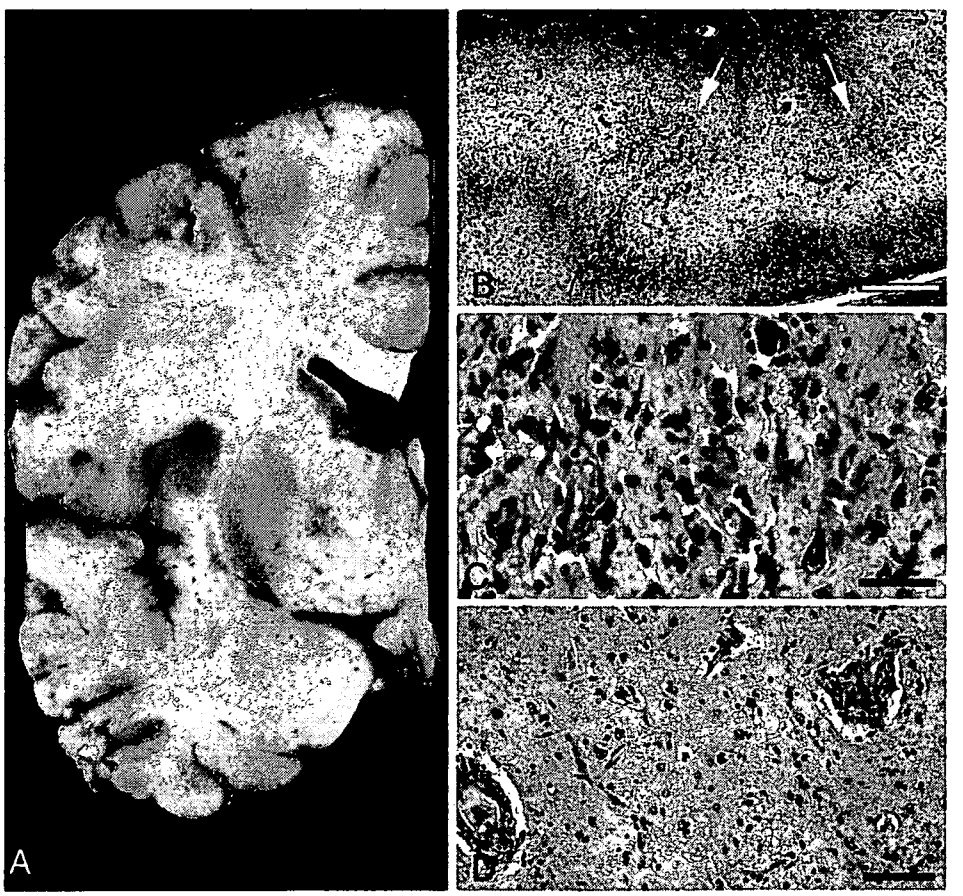

Fig. 2. Neuropathological findings: (A) coronal slice through the left cerebrum. No lesions visible on macroscopic examination; (B) foci of neuronal loss (arrows) surrounded by spongy state in the rostral CA1 of hippocampus. Klüver–Barrera staining (Bar 500 μm); (C) foci of neuronal loss and neuronophagia in the rostral CA1 of hippocampus. Hematoxylin and eosin (HE) staining (Bar 50 μm) and (D) neuronal loss, fibrillary astrocytosis and lymphocytic perivascular cuffing were seen in the rostral part of amygdala. HE staining (bar 50 μm).

failed to be controlled with propofol and acyclovir in addition to the anticonvulsants. An electroencephalogram revealed multifocal spikes without periodic synchronous discharges and periodic lateralized epileptiform discharges. Autoantibodies, including antinuclear antibody, anti-SS-A/B antibodies, and anti-Hu antibodies were all negative. Autoantibody against the GluR IgM-ε2 [11] in the serum was positive, autoantibody against GluR IgG-δ2 in the CSF was positive, and VGKC antibody and P/Q-type voltage-gated calcium channel antibodies were negative in the serum and CSF. Antibodies for several viruses including HSV in the serum and CSF were negative 10 days after the onset of seizures. She had acute renal failure complications and died 12 days after the onset of the seizures.

The direct cause of death was acute renal tubular necrosis and purulent pneumonia. Both laboratory data and pathological examination revealed that the patient did not have any malignant tumors or collagen disease. The brain, weighing 1183 g, was macroscopically unremarkable except for mild swelling (Fig. 2A). Microscopically, there were no leptomeningitis. The amygdala and hippocampus showed small foci of neuronal loss with neuronophagias, proliferation of microglias and hypertrophic astrocytes (Fig. 2B and C). These foci were surrounded by a spongy state. Only a few lymphocytic perivascular cuffings occurred in the amygdala (Fig. 2D). No intranuclear inclusion bodies were found anywhere. Immunohistochemistry for HSV-1, -2 and human herpesvirus-6 was negative. No tissue necrosis or haemorrhage were found in the cerebral cortex including the cingulate, insular, and parahippocampal cortex.

Besides demonstrating evidence of HSV infection, HSV encephalitis shows extensive necrosis with haemorrhage in the medial temporal lobe, insular and cingulate gyri bilaterally [6], where the brain MRI shows high signal intensities. Furthermore, the lesions are bilateral, but not always symmetrical in distribution. In our case, however, abnormal signal intensities were limited in the hippocampus and amygdala bilaterally and symmetrically. No haemorrhagic necrosis was found anywhere, even though there would not have been sufficient time for our patient to develop it. Finally, there were no intranuclear inclusions or immunohistological evidence of HSV infection.

Recently, it is suggested that the presence of autoantibodies against the GluR-ε2 in the CSF of non-herpetic acute encephalitis involves in autoimmune pathogenic mechanism [3,9]. In the CSF of this patient, autoantibody against the GluR-ε2 was negative, while the autoantibody against the GluR-δ2, which is against cerebellar Purkinje cell-specific antibody [11] was positive. The other similar cases as shown in Table 1 [1,4,5,7,8,10] were not examined for the presence of these antibodies. Unfortunately, it remains obscure that this antibody played a role in development of the disease in our case.

There has been only one pathological report of a patient similar to our case: a 53-year-old woman who died 36 days after the onset of illness, and showed neuronal loss in the hippocampus, and neuronophagia and gliosis in the amygdala [7]. As seen in the present patient, this patient showed no evidence of HSV infection, no apparent necrosis in the brain, and the

Table 1
Clinical characteristics and MRI abnormalities of patients with non-herpetic acute limbic encephalitis

Patients	Kohira et al. [4]	Kusuhara et al. [5]				Asaoka et al. [1]						Nonaka et al. [8]	Takahashi et al. [10]		Maki et al. [7]	Present case
		Case 1	Case 2	Case 3	Case 4	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6		Case 1	Case 2		
Age (year)	25	40	38	38	53	34	60	73	35	23	18	42	25	58	53	59
Sex	M	M	F	M	F	M	F	F	M	M	F	F	F	M	F	F
Clinical symptoms																
Impaired consciousness	2+	3+	3+	+	3+	2+	+	2+	2+	2+	2+	3+	+	+	3+	3+
Seizures	2+	2+	3+	+	–	+	+	+	2+	+	3+	3+	2+	–	3+	3+
Cerebrospinal fluid																
Cells (mm^3)	52	17	47	9	10	10	5	32	8	5	320	10	1	76	normal	10
Protein(mg/dl)	25	325	55	27	50	72	32	29	41	28	86	40	15	45	normal	50
MRI abnormalities																
Hippocampi	B	B	B	B	L	B	R>L	B	B	B	L>R	B	B	B	B	B
Amygdalae	B	B	B	B	L	B	R>L	B	B	B	L>R	B	B	B	B	B
Cingulate gyri	–	–	L	–	–	–	–	B	–	–	–	–	–	–	–	–
Sequelae	+	+	+	+	+	+	2+	2+	+	+	+	+	+	+	died	died

M: male; F: female; B: bilateral; L: left; R: right; (–): negative; (+): mild; (2+): moderate and (3+): severe.

lesions were exclusively limited to the hippocampus and amygdala. In this regard, similar clinical cases with acute encephalitis have accumulated in Japan, as shown in Table 1 [1,4,5,7,8,10]. Many cases with this type of encephalitis showed good prognosis, although patients died because of uncontrollable generalized seizures during the clinical course. It is likely that our case showed the neuropathological changes of non-herpetic acute limbic encephalitis as a possible clinicopathological new entity.

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Short communication

Voltage-gated potassium channel antibodies associated limbic encephalitis in a patient with invasive thymoma

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Abstract

Recently, limbic encephalitis (LE) associated with Voltage-gated potassium channel antibody (VGKC-Ab) has been postulated as a new autoimmune disorder. Most previously reported cases of VGKC-Ab-associated LE were non-paraneoplastic, and reports of a paraneoplastic type are rare. Here we describe a 59-year-old woman with paraneoplastic VGKC-Ab-associated LE preceding the recurrence of invasive thymoma. There was a close temporal relationship between the clinical course and the changes of the VGKC-Ab titer. Unlike many of the non-paraneoplastic VGKC-Ab-associated LE cases, our cases showed the more extensive high intensity lesions on MRI and the absence of seizure and hyponatremia.

Keywords: Voltage-gated potassium channel antibody; Limbic encephalitis; Invasive thymoma

1. Introduction

Limbic encephalitis (LE) is clinically characterized by the subacute onset of disorientation, psychiatric symptoms, and impairment of short-term memory. Various conditions have been suggested to be associated with LE. Two recent reports have described the features of the non-paraneoplastic type of LE associated with voltage-gated potassium channel antibodies (VGKC-Ab) [1,2]. Here we describe a case of paraneoplastic VGKC-Ab-associated LE in a patient with invasive thymoma. This is the first reported Japanese case.

2. Case report

A 59-year-old right-handed retired local government official was referred to our hospital with a 3-day history of memory impairment. At the age of 56 years, invasive thymoma had been diagnosed and was treated with transsternal extended thymectomy and postoperative radiation therapy. At the age of 57 years, herpes zoster affected the right upper limb. The patient also suffered from post-herpetic neuralgia and was under treatment with carbamazepine. She was also taking antidiabetic and antihypertensive medications. She was a non-smoker and had no history of alcohol intake.

On admission, the vital signs were normal. Her short-term memory was impaired. The Mini-Mental State Examination (MMSE) score was 19/30, and she was disoriented in time. Her daughter reported that the patient had become apathetic. She had no symptom or signs of neuromyotonia; electromyography was not performed. There was no history of seizures and no excessive sweating.

Laboratory tests demonstrated transient leukocytosis (12,000/mm^3), an increased gamma-glutaryl transpeptidase level, and hyperglycemia (280 mg/dl), but other investigations yielded normal results. Serum sodium was also normal. Screening for autoantibodies (including thyroperoxidase antibodies) was negative, except for single-stranded DNA antibodies. Acetylcholine receptor antibodies and Hu and Yo

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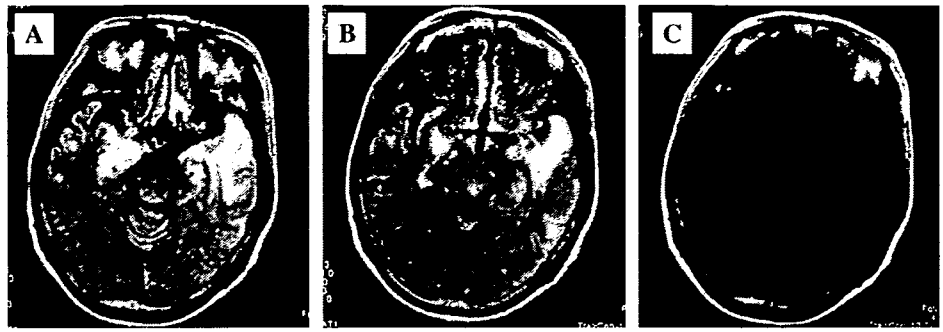

Fig. 1. Initial MRI findings. FLAIR images demonstrate bilateral hyperintense areas in the hippocampus and parahippocampal gyrus, as well as a mass effect, especially on the left side. High signal intensity lesions are also present in the right insular cortex, as well as bilaterally in the frontobasal cortex and cingulate gyrus. (A, B) On T1-weighted image, the lesions were demonstrated low intensity. (C).

paraneoplastic antibodies were negative. Cerebrospinal fluid examination (including PCR for herpes simplex virus and herpes zoster virus) yielded normal results, except for mild elevation of the protein content (60 mg/ml). The EEG was normal. Her initial chest computed tomography (CT) scan demonstrated no evidence of recurrent thymoma. ^{67}Ga scintigraphy and CT scans of the abdomen and pelvis also failed to show any evidence of malignancy. Brain magnetic resonance imaging (MRI) was done at 3 days after the onset. T2-weighted and fluid-attenuation inversion recovery (FLAIR) images demonstrated bilateral hyperintense areas in the medial part of the temporal lobe. High intensity lesions were also seen in the right insular cortex, as well as bilaterally in the frontobasal cortex and cingulate gyrus (Fig. 1).

Over the next 10 days, there was improvement of her memory and MMSE (to 27/30). At discharge, 3 weeks after the onset, repeat MRI showed attenuation of the bilateral medial temporal lobe signal changes, especially on the right side. Hyper-intensity of the superficial cortical layers was visible on T1-weighted images. An immunosuppression therapy was advised but refused by the patient and her family.

Six months later, her memory began to decline again and memory impairment was gradually progressive. Ten months after the onset of her illness, repeat chest CT demonstrated the recurrence of invasive thymoma. At that time, the MMSE score was 23/30. On brain MRI, the lesions in the temporal lobes were smaller, but those in the frontobasal lobes were larger, and profound hippocampal atrophy was shown (Fig. 2). She consented to receive chemotherapy for thymoma and was referred to the department of respiratory disease. She was treated by combination of carboplatin and etoposide. Two months after the recurrence, her mental state partial improved with diminishment of thymoma. Retrospective analysis of serum samples obtained at three times (onset, remission, and recurrence) showed high titers of VGKC antibodies by radioimmunoassay using whole rabbit brain homogenate, as described previously.[6] The VGKC antibody titer was 403 pM at onset, 373 pM at remission, 917 pM at recurrence, and 649 pM after chemotherapy (normal range <100).

3. Discussion

The most prominent clinical feature in this patient was cognitive dysfunction that caused anterograde amnesia with a remitting and relapsing course. Her signs and symptoms, as well as the MRI findings, were typical of limbic encephalitis. The interesting finding in our patient was the existence of VGKC-Ab, which showed a temporal relationship with her LE symptoms and with the recurrence of thymoma. Recently, a new type of LE associated with VGKC-Ab has been proposed [1,2]. The main features of this condition are memory disturbance, confusion, seizures, and hyponatremia [1,2], while neuromyotonia has only been found in one patient. Compared with non-paraneoplastic VGKC-Ab-associated LE, there have been few reports about paraneoplastic VGKC-Ab-associated LE – only seven patients with thymoma [3,4] and two with lung cancer [5] have been reported so far. Detailed clinical features have only been described for one case of thymoma with MG [3]. Some features of our case differ from the previously reported non-paraneoplastic type of LE cases, although the most of clinical features of our patient was similar. One of the features was the more extensive high intensity lesions on MRI, involving the right insular cortex, both frontobasal cortices and cingulate

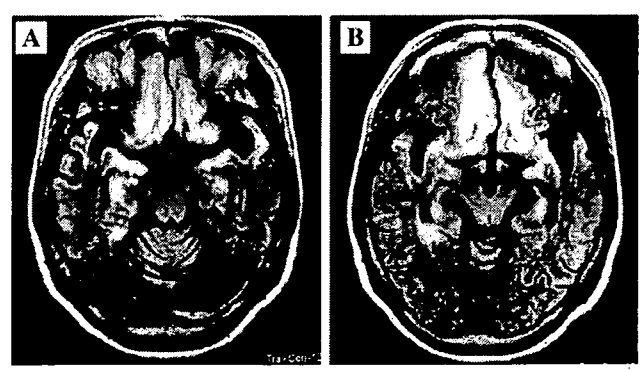

Fig. 2. Follow-up MRI (recurrence at 10 months after the onset) findings. FLAIR images show improvement of the lesions in the bilateral temporal lobes (A), but there is expansion of hyperintense areas in the bilateral frontobasal lobes. (B).

gyrus. Unlike many of the non-paraneoplastic VGKC-Ab-associated LE cases, the serum sodium was normal, and the patient did not develop seizures, which may be relevance. The titer of the VGKC antibodies of this case was not extremely high, starting at around 400 pM, and becoming 900 pM at recurrence of the tumor. Levels around 400 pM may be more common in cases associated with tumors than in the non-paraneoplastic forms [4].

When VGKC-Ab are detected in patients with paraneoplastic neurological syndromes other than LE, thymoma is found in the majority of cases [3,4,7–9]. VGKC-Ab is also found in 13% of thymoma patients without neurological disorders [4]. It is thought that the following two disorders are often related to VGKC-Ab and can coexist with thymoma. One condition is acquired peripheral nerve hyperexcitability (APNH), including neuromyotonia, cramp-fasciculation syndrome, and Isaac's syndrome [9]. The other is Morvan's syndrome, a rare condition that features acquired neuromyotonia, autonomic disturbance, hypersecretion, and cognitive impairment [10,11]. Our patients showed spontaneous and treatment-related recovery of clinical findings with the decreasing of the VGKC antibody titer and relapse with the recurrence of invasive thymoma and strikingly increasing anti body titer. Spontaneous recovery of paraneoplastic limbic encephalitis is unusual. This clinical course suggested that VGKC-Ab of our patient related to occurrence of limbic encephalitis and thymoma, although they are detected and found in thymoma patients without neurological symptom [4].

Antibody-mediated VGKC dysfunction is thought to cause hyperexcitability of peripheral motor axons and nerve terminals in patients with APNH [9,12,13]. In VGKC-Ab-associated LE, previous reports [1–3] and the findings in our patient have demonstrated a temporal relationship between the clinical course and changes of the VGKC-Ab titer. Immunohistochemistry using serum samples from a patient with VGKC-Ab and limbic encephalitis showed strong immunostaining of the molecular layer of the dentate gyrus [3]. It can be suggested that VGKC-Ab plays a pathogenic role in VGKC-Ab-associated LE based on these findings.

Further studies are required to confirm that VGKC antibodies cause limbic encephalitis. LE associated with thymoma may represent a form of VGKC-Ab-mediated autoimmune ion channelopathy, as well as APNH and Morvan's syndrome, and it might show a good response to immunotherapy similar to myasthenia gravis and other autoimmune disorders.

Acknowledgement

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Short communication

Acute encephalopathy with refractory status epilepticus: Bilateral mesial temporal and claustral lesions, associated with a peripheral marker of oxidative DNA damage

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Abstract

We describe a 12-year-old girl, who had been medicated with theophylline for bronchial asthma and developed acute encephalopathy with refractory status epilepticus, showing bilateral mesial temporal and claustral lesions, which were evident on fluid-attenuated inversion recovery images, obtained with 1.5 T magnetic resonance imaging. To date, oxidative stress has been implicated in aging or various disorders, including inflammatory or degenerative neurological disorders. One of the oxidative stress markers, 8-hydroxydeoxyguanosine, was increased in our patient's cerebro-spinal fluid, plasma and urine. We speculate that augmented oxidative stress was associated with refractory status epilepticus in our patient, accompanying bilateral mesial temporal, claustral lesions and severe neuronal damage. Serial measurements of oxidative stress markers in acute encephalitis, encephalopathy, or status epilepticus could clarify the relationships between acute brain damage and free radicals.

Keywords: Acute encephalopathy; Status epilepticus; Theophylline; 8-hydroxydeoxyguanosine; Oxidative stress; Claustrum

1. Introduction

Acute encephalopathy or encephalitis presenting with refractory status epilepticus is rare, but results in severe mortality, and the known etiologies are diverse, including infection, metabolic derangement, hypoxia–ischemia, and intoxication. However, a certain part of the pathophysiology remains an enigma [1–3]. To date, oxidative damage has been implicated in aging or various disorders, including inflammatory or degenerative neurological disorders [4]. We report a patient with acute encephalopathy presenting with refractory status epilepticus, showing bilateral mesial temporal and claustral lesions, which could be related to DNA oxidative damage.

2. Case report

A 12-year-old girl developed frequent tonic convulsions and was admitted to our hospital after mild drowsiness and fever for 7 days. Since 9 years of age, she had been medicated with theophylline (300 mg/day) with a blood level of 6.7 μg/ml for bronchial asthma. There was no history of neurological disorders nor any contributory family histories.

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She showed consciousness disturbance without focal neurological signs. Routine hematology, blood chemistry, cranial computed tomography and magnetic resonance imaging (MRI) on day 7 of illness did not detect any abnormalities. Cerebro-spinal fluid (CSF) examinations on day 7 and 10 yielded normal findings, including cell count, glucose, protein, bacterial and viral cultures, polymerase chain reactions for herpes simplex and enteroviruses, and autoantibodies against the glutamate receptors, except for elevations of cytokines; interleukin-2, 4 and 6 were 5.0 pg/ml (normal control<4.6), 13.9 pg/ml (normal control<11.6) and 83.9 pg/ml (normal control<9.7), respectively. Oxidative stress marker, 8-hydroxydeoxyguanosine (8-OHdG) was measured with a commercial enzyme-linked immunosorbent assay kit (highly sensitive 8-OHdG check; Japan Institute for the Control of Aging, Shizuoka, Japan. More information is available at http://www.jaica.com/biotech/) [5]. The levels of 8-OHdG concentration in CSF on day 10, plasma on day 8 and 17 were 0.070, not detected and 0.245 ng/ml (not detected in normal control), respectively. Urine 8-OHdG/creatinine ratio was 36.6 ng/mg creatinine (normal control 12.1±5.5, mean±SD) on day 23 [6,7]. Because 8-OHdG was measured retrospectively with stored samples, data from other days were not available. Although the fever subsided, seizures progressed to an intractable status; focal clonic convulsions involving the face or extremities generalized after intervals of a few minutes. Electroencephalography showed multifocal spikes or sharp waves, tending to burst. Conventional anti-epileptic drugs, including phenobarbital, phenytoin and valproate, and anesthetic agents, including midazolam, thiamylal and propofol, failed. Thiopental alleviated seizures, but withdrawal resulted in seizure recurrence. Cranial MRI on day 27 demonstrated bilateral mesial temporal and claustral lesions (Fig. 1). Thiopental was stopped on day 82 due to side effects, such as leukopenia, hypotension, atelectasis, thrombophlebitis, renal failure and liver failure. Thereafter,

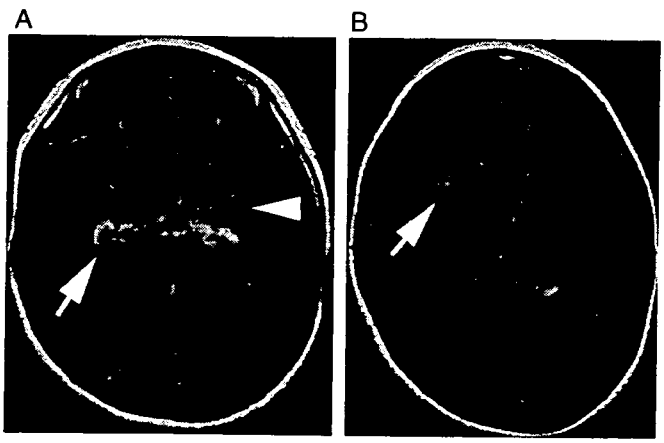

Fig. 1. Axial fluid-attenuated inversion recovery (FLAIR) images (TR/TE/TI 6000/120/2100 ms), using a 1.5 T imager, on day 27 showed high signal lesions in both hippocampi (arrow), amygdalae (arrowhead) (A), and claustrum (arrow) (B).

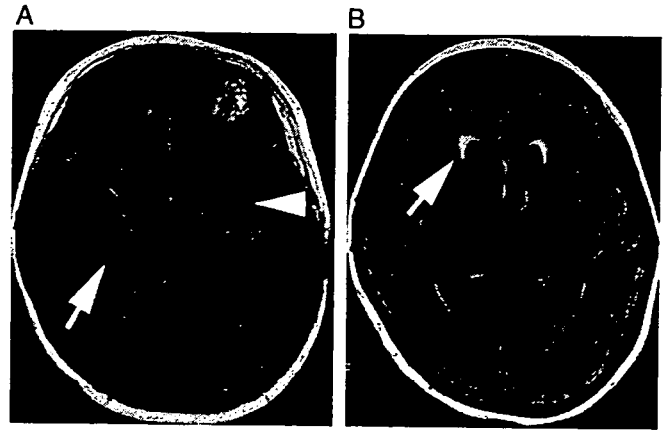

Fig. 2. Axial FLAIR images (TR/TE/TI 8002/104/2000 ms), using a 1.5 T imager, on day 101 showed high signal lesions in both hippocampi (arrow), amygdalae (arrowhead) (A) and ventricular rims (arrow) (B). Cerebellar and cerebral atrophy were evident.

phenytoin and high-dose phenobarbital were administered to reduce seizures. Cranial MRI on day 101 showed severe brain atrophy, and bilateral mesial temporal lesions (Fig. 2). Seven months after the onset, she could not speak or sit alone.

3. Discussion

Deoxyguanosine is one of the constituents of DNA and when it is oxidized, it is altered to 8-OHdG, which is considered a key biomarker of oxidative DNA damage [4]. Furthermore, 8-OHdG is increased in aging or diverse disorders, such as neoplasm, neurodegenerative disorders, cardiovascular disease, diabetes mellitus and inflammatory disorders [4,5,8]. Moreover, 8-OHdG is increased within the brain, including the amygdala and hippocampus, in the seizure-induced rat and it is associated with increased DNA fragmentation, resulting in neuronal cell death [9]. The high levels of 8-OHdG concentrations in our patient's CSF, plasma and urine, indicate that oxidative stress plays a key role in our patients, resulting in severe neuronal damage.

Claustrum, a thin sheet of gray matter between the insula and putamen, is affected in several disorders. However, the pathophysiology of these disorders remains obscure [10]. We hypothesize that oxidative stress plays some role in claustral lesions. Claustrum as well as the cortex, amygdala and hippocampus, contains nitric oxide-producing neurons, which were involved in our patient [11,12]. Neurotoxicity in rats with kainic acid-induced status epileptics is associated with increments in nitric oxide, possibly resulting in augmented oxidative stress in these regions [12]. Other studies also show that disorders involving the claustrum, such as ischemia–hypoxia, Wilson's disease, mitochondrial disorders, and encephalitis are associated with augmented oxidative stress [10,13–16].

Theophylline affects the seizure threshold, and we speculate that theophylline was associated with a protracted course in our patient [17]. Although, the theophylline blood

level in our patient was within the accepted therapeutic range, Delanty et al. reported that a surprisingly high rate of status epilepticus patients (eleven out of 41; 27%) were taking theophylline at the time status epilepticus developed and all except one had shown standard therapeutic blood levels [18]. Theophylline-induced seizures in mice may be associated with free radicals, which also suggests a causative role of augmented oxidative stress in our patient [19]. Drugs other than theophylline, including anti-epileptic drugs and anesthetic agents, could affect oxidative stress, however, their effects were not one-way; some might augment oxidative stress, others could reduce it [20–24].

There are some difficulties with chronological analysis among symptoms, cranial MRI findings, and 8-OHdG levels, because 8-OHdG serial examinations were lacking and MRI examinations had long intervals. Convulsions started on day 7, but cranial MRI on day 7 revealed no abnormalities, plasma 8-OHdG was not detected on day 8, CSF 8-OHdG was detected on day 10, urinary 8-OHdG was high on day 23, and cranial MRI on day 27 demonstrated bilateral mesial temporal and claustral lesions. In rat experimental models, systemic kainic acid, which induces seizures, increased cerebral 8-OHdG levels, up to seven-fold within 72 h [9]. This is compatible with our patient's course. Oxidative DNA damage could be evoked within a few days after status epileptics and neuroimaging changes may continue for some time beyond this.

In conclusion, it is possible that oxidative stress was associated with refractory status epilepticus in our patients, accompanying bilateral mesial temporal, claustral lesions and severe neuronal damage, which is partly due to theophylline. There may be a vicious cycle; refractory status epilepticus could result in limbic lesions and limbic lesions could worsen the refractoriness of status epilepticus itself. Serial measurements of oxidative stress markers, including 8-OHdG in acute encephalitis, encephalopathy or status epilepticus could clarify the relationships between acute brain damage and free radicals.

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Development of the loop-mediated isothermal amplification method for rapid detection of cytomegalovirus DNA

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Abstract

Cytomegalovirus (CMV) loop-mediated isothermal amplification (LAMP) was performed on DNA extracted from CMV (AD-169)-, herpes simplex virus (HSV) 1 (KOS)-, HSV-2 (186)-, varicella-zoster virus (Oka-vaccine)-, human herpesvirus (HHV)-6 A (U1102)-, HHV-6 B (Z29)-, and HHV-7 (RK)-infected cells. Although amplified CMV demonstrated typical ladder patterns, no LAMP product was detected in reactions performed with other viral DNAs. The sensitivity of the CMV LAMP was 500 copies/tube, as determined by either agarose gel electrophoresis or turbidity assay. To determine whether CMV LAMP could be used for quantitative analysis of viral DNA, threshold times, defined as the time (in seconds) to reach the threshold level (0.1), were measured by amplification of serial dilutions of the plasmid DNA. The standard curve exhibited a correlation coefficient of 0.944, a slope of −208.1, and a *y*-intercept of 3261.4. Following these initial validation experiments, we analyzed 180 samples collected serially from 20 pediatric hematopoietic stem cell transplant recipients. Detection of CMV DNA in whole blood (WB) was tested by CMV LAMP and real-time polymerase chain reaction (PCR). When >500 copies/tube (>5000 copies/200 μl of WB) was defined as positive for CMV infection, the sensitivity, specificity, positive predictive value, and negative predictive values of the CMV LAMP were 80.0, 98.9, 66.7, and 99.4%, respectively.

Keywords: CMV; LAMP; Real-time PCR; Immunocompromised patient

1. Introduction

Primary cytomegalovirus (CMV) infection occurs in subclinical fashion in early childhood with subsequent lifelong latent infection. Up to 80% of healthy adults in western countries are seropositive, indicating established latency with a capability for viral reactivation. The mechanism of reactivation is not fully understood, but appears strongly related to impaired immunity to the virus. For this reason, CMV is one of the most common opportunistic pathogens in hematopoietic stem cell and solid-organ transplant recipients and in AIDS patients. Clinical manifestations of the virus (CMV diseases) vary in patients from benign to severe, life-threatening clinical courses. The administration of antiviral therapy for CMV, based on evidence of CMV infection, has become a common strategy in treating the disease (Ljungman, 2002; Meijer et al., 2003). Thus, reliable and rapid diagnostic procedures are critical for effective patient management. To date, however, rapid virological diagnosis has proven difficult, as isolation of the virus and serological testing require substantial time to obtain definitive results. The pp65 antigenemia assay has largely replaced viral isolation and shell vial assays for CMV surveillance due to its improved sensitivity, predictive value, and rapidity. However, the limitations of the antigenemia assay for detecting reactivation are the costs of technician time, the need for technical expertise, the inability to test stored blood, and the requirement for neutrophil counts $>0.5 \times 10^9$ cells/l. Meanwhile, quantitative polymerase chain reaction (PCR) is a valuable tool for monitoring active viral

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