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detected higher incidence rates of occult bacteremia and of IPD overall than were found by community physicians. The baseline incidence rate of IPD in Vancouver (235.7 per 100,000 for 6- to 23-month-old children) was much higher than rates previously reported for urban populations across Canada,[6] which featured in estimations of the cost-effectiveness of PCV7 programs.[9,10] If disease rates were underestimated, the cost-effectiveness of vaccination would have been underestimated as well.

Although this study featured robust case-finding in a large pediatric population, it had some limitations. PCV7 was available for private purchase for 2 years before program implementation, and so the baseline rate of IPD might have been under-estimated. However, no decrease in incidence rates was evident during the 2 preprogram years. The extent of private vaccine use is unknown. Serotype data were lacking for some isolates, and so the rate reduction for serotypes included in the vaccine was estimated rather than directly observed. Immunization histories were missing for some cases. The 2-year observation period after program introduction was likely too short to measure the full impact of vaccination, including indirect protection of other age groups.

We conclude that PCV7 infant vaccination in BC has been highly effective.

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MATRIX METALLOPROTEINASE-9 AND TISSUE INHIBITORS OF METALLOPROTEINASES 1 IN INFLUENZA-ASSOCIATED ENCEPHALOPATHY

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Abstract: Matrix metalloproteinase-9 (MMP-9) and tissue inhibitors of metalloproteinases 1 (TIMP-1) play important roles in the function of the blood–brain barrier. Serum MMP-9 and TIMP-1 concentrations were determined in influenza virus infection with or without neurologic complications. Our results suggest that an imbalance between MMP-9 and TIMP-1 damages the blood–brain barrier and promotes febrile seizure or encephalopathy in influenza virus infection.

Key Words: Epstein–Barr virus, influenza-associated encephalopathy, MMP-9, respiratory syncytial virus, TIMP-1

Accepted for publication January 19, 2007.

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This study was supported by grants from the Ministry of Health, Labor and Welfare (H18-Shinkou-6) and the Ministry of Education, Culture, Sports, Science and Technology (A-17209037).

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DOI: 10.1097/INF.0b013e31803994a0

Matrix metalloproteinases (MMPs) constitute a family of zinc-binding proteinases that degrade the extracellular matrix proteins.[1] MMP-9 is mainly produced by inflammatory cells such as T cells and macrophages, and is capable of degrading collagen IV, a major component of the basement membrane of the cerebral epithelium and responsible for the integrity of the blood–brain barrier.[2] The activity of MMPs is further controlled by specific tissue inhibitors of metalloproteinases (TIMPs), and TIMP-1 has a high avidity for MMP-9.[3]

Influenza-associated encephalopathy is a severe neurologic complication of influenza manifesting seizures and progressive coma with a high-grade fever.[4] The pathogenesis of influenza-associated encephalopathy remains unclear. We have reported that several proinflammatory cytokines are elevated in serum and cerebrospinal fluid in patients with influenza-associated encephalopathy and are related to the clinical severity of the disease.[5–8] Brain computed tomography abnormalities, such as brain edema and low densities in localized areas, are associated with a poor prognosis in these patients.[4] A postmortem examination of 1 fatal case of influenza-associated encephalopathy revealed vasogenic brain edema, suggesting that the vascular endothelial cells were impaired.[9] It is possible that the blood–brain barrier is damaged in influenza-associated encephalopathy.

To investigate the role of MMP-9 and TIMP-1 in the pathogenesis of influenza-associated encephalopathy, we determined the relationship between serum concentrations of MMP-9 and TIMP-1 in patients with influenza virus infection with or without neurologic complications.

SUBJECTS AND METHODS

Informed consent was obtained from the parents of the patients and controls enrolled in this study. Serum samples were obtained from influenza-associated encephalopathy patients on admission to Yamaguchi University Hospital and 11 research cooperation hospitals in Japan, from December 1999 to May 2005, and were obtained from the patients with influenza-associated febrile seizures, uncomplicated influenza, Epstein–Barr virus (EBV) infection and respiratory syncytial virus (RSV) infection on admission to Yamaguchi University Hospital from March 2003 to January 2006. The specimens were stored frozen at −80°C until assay.

Influenza-Associated Encephalopathy, Influenza-Associated Febrile Seizures and Uncomplicated Influenza. The criteria for the diagnosis and the day of onset of influenza-associated encephalopathy, influ-

enza-associated febrile seizures and uncomplicated influenza were described in our previous article.[8] Thirty-five patients were enrolled with influenza-associated encephalopathy (22 males and 13 females, aged from 6 months to 19 years: mean, 5.8 years). These patients were divided into 2 groups, ie, those who died (n = 7 of 17) or developed neurologic sequelae (n = 10 of 17), and those who had no sequelae of follow-up (n = 18). Serum samples were obtained from these patients on day 1.0 ± 0.5 (range, 1–3) of the illness.

Thirteen patients were enrolled with influenza-associated febrile seizures (8 males and 5 females, aged from 2 to 10 years: mean, 5.4 years). Serum samples were obtained on day 1.0 ± 0.3 (range, 1–2) of the illness.

Forty-six patients were enrolled with uncomplicated influenza (26 males and 20 females, aged from 11 months to 10 years: mean, 5.2 years), and serum were obtained on days 2.0 ± 1.0 (range, 1–4) of the illness.

Control Subjects. Control subjects comprised 8 patients who had EBV infection (3 males and 5 females, aged from 2 to 12 years: mean, 5.8 years) and 37 patients who had RSV infection (21 males and 16 females, aged from 1 to 19 months: mean, 0.7 years). Serum were obtained from the patients with EBV infection on days 7.0 ± 3.5 (range, 2–13) and those with RSV infection on days 4.0 ± 2.5 (range, 2–7) of the illness. Thirty-three healthy children (15 males and 18 females, aged from 2 to 15 years: mean, 6.4 years) also served as controls.

Determination of MMP-9 and TIMP-1 Concentrations. The serum concentrations of MMP-9 and TIMP-1 were determined with sandwich-type ELISA kits (Amersham, Buckinghamshire, UK). Assays were performed following the manufacturer's instructions. The detection limits were 2.5 ng/mL for MMP-9 and 2.4 ng/mL for TIMP-1.

Statistical Analysis. All values are medians ± SD. The differences in the results between groups were analyzed using the Mann–Whitney *U* test.

RESULTS

Serum MMP-9 concentrations in influenza-associated encephalopathy with poor prognosis, influenza-associated febrile seizures and uncomplicated influenza were significantly higher than the healthy controls ($P < 0.0001$, $P = 0.0005$, $P < 0.0001$, respectively) (Fig. 1). The serum MMP-9 concentrations in influenza-associated encephalopathy with poor prognosis were significantly higher than those for uncomplicated influenza, and the healthy controls ($P = 0.0149$, and $P < 0.0001$, respectively).

Serum TIMP-1 concentrations in influenza-associated encephalopathy with poor prognosis were significantly higher than those in influenza-associated encephalopathy without sequelae, influenza-associated febrile seizures and the healthy controls ($P = 0.0087$, 0.018 and 0.0062, respectively). The serum TIMP-1 concentrations in influenza-associated encephalopathy without sequelae and influenza-associated febrile seizures were significantly lower than those in uncomplicated influenza ($P < 0.0001$ and <0.0001, respectively). The MMP-9/TIMP-1 ratios in influenza-associated encephalopathy with poor prognosis, influenza-associated encephalopathy without sequelae and influenza-associated febrile seizures were significantly higher than those for uncomplicated influenza ($P = 0.0099$, 0.0079 and 0.0053, respectively) and the healthy controls ($P = 0.0134$, 0.012 and 0.0093, respectively).

The serum MMP-9 concentrations and MMP-9/TIMP-1 ratios for uncomplicated influenza were significantly higher than those in EBV infection ($P = 0.0006$ and $P < 0.0001$, respectively) and RSV infection ($P = 0.0002$ and $P < 0.0001$, respectively), and serum TIMP-1 concentrations were significantly lower than those in EBV infection ($P = 0.0001$) and RSV infection ($P = 0.0017$).

DISCUSSION

Results from our present study suggest that MMP-9 and TIMP-1 are related to the pathogenesis of influenza-associated encephalopathy on the basis of the elevated serum MMP-9 and MMP-9/TIMP-1 ratios, and associated with the clinical severity of influenza-associated encephalopathy. The serum MMP-9 levels and MMP-9/TIMP-1 ratios for uncomplicated influenza were significantly higher than those in EBV and RSV infections, and the serum TIMP-1 concentrations for uncomplicated influenza were significantly lower. These findings suggest that TIMP-1 was not sufficiently produced in influenza virus infection compared with RSV and EBV infections, though MMP-9 was abundantly produced in influenza virus infection. However, EBV and RSV infections may be inappropriate disease controls. EBV infection affects lymphocytes, but not airway epithelial cells, and RSV infects young infants. Another hypothesis is that TIMP-1 is overly consumed in influenza virus infection and, as a result, the serum TIMP-1 values are not elevated. In vitro, influenza virus produced MMP-9, but had little effect on TIMP-1, in cultured cells.[10] Moreover, the serum TIMP-1 levels in influenza-associated encephalopathy without sequelae and influenza-associated febrile seizures were significantly lower than those for uncomplicated influenza. One hypothesis is that patients with neurologic complications of influenza could not produce a sufficient amount of TIMP-1. The serum TIMP-1 concentrations in influenza-associated encephalopathy with poor prognosis were not lower than those for the Flu. Interleukin-10 (IL-10) is markedly elevated in the serum of influenza-associated encephalopathy with poor prognosis.[7,8] IL-10 stimulates TIMP-1 production.[3] We hy-

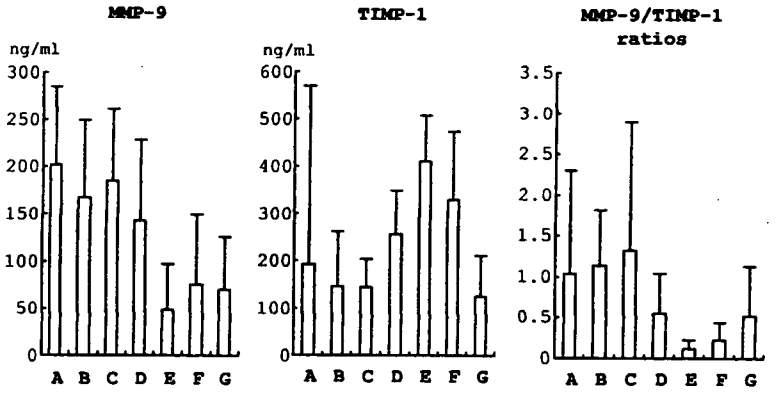

FIGURE 1. Serum concentrations of MMP-9, TIMP-1 and the ratios of MMP-9/TIMP-1 in influenza-associated encephalopathy with poor prognosis (A), influenza-associated encephalopathy without sequelae (B), influenza-associated febrile seizures (C), uncomplicated influenza (D), EBV infection (E), RSV infection (F)and the healthy controls (G). Data are presented as medians + 1 SD.

pothesize that the influenza-associated encephalopathy patients with poor prognoses could not produce a sufficient amount of TIMP-1, but a large amount of IL-10 strongly stimulated TIMP-1 production.

In summary, an imbalance between MMP-9 and TIMP-1 occurred in influenza virus infection, especially with neurologic complications. The imbalance may damage the blood–brain barrier and promote febrile seizure or encephalopathy.

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PREVALENCE OF NASAL COLONIZATION WITH METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) IN CHILDREN *A MULTICENTER CROSS-SECTIONAL STUDY*

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Abstract: In this cross-sectional multicenter study, we determined the rate of nasal carriage of methicillin-resistant *Staphylococcus aureus* (MRSA) in children admitted to 9 training hospitals in Switzerland during 1 month. From 1337 patients, 1363 nasal swabs were obtained (mean age 6.1 years, median 4.7 years, interquartile range 1.3–10.4 years) and 562 (41.3%) grew *S. aureus*. Only one isolate was MRSA (0.18%) which encoded *mecA* and *femA* genes as well as SCC*mec* type IV, whereas Panton-Valentine leukocidin (PVL) was absent.

Key Words: *Staphyloccus aureus*, MRSA, nasal carriage, children
Accepted for publication February 16, 2007.

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DOI: 10.1097/INF.0b013e31804d244a

Staphylococcus aureus is an important pathogen which causes community-associated and health care-associated infections in all age groups, including children world wide.[1–3] The clinical spectrum of disease comprises infections of the skin, bones and soft tissue, respiratory tract, blood and central nervous system. Resistance to methicillin was first described in *S. aureus* isolates in the early 1960s in England,[4] and has emerged in many countries since.[1] Methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) colonize the skin and mucosae of humans, but the anterior nares is the most frequent site of carriage.[5] Pooled data from 10 studies, mostly from the United States, consisting of 8350 children and adults revealed an overall prevalence of nasal colonization with MRSA of 1.3% and 0.2%, respectively, when persons with health care-associated risk factors were excluded.[3] Some data on the frequency of nasal MRSA colonization are available in children.[3,6–11]

However, this is a dynamic process, and as such ongoing studies are critical in understanding of MRSA epidemiology.

The goal of our study was to determine the carriage frequency of *S. aureus* and MRSA in particular in children admitted to 9 training hospitals in Switzerland, and to evaluate possible risk factors associated with MRSA colonization.

PATIENTS AND METHODS

This was a multicenter, cross-sectional study in children admitted to Swiss children's hospitals during a 1-month surveillance period, March 16 to April 15, 2006. Study sites were 9 children's hospitals representing all geographical areas, and included all 5 university hospitals of Switzerland. Children 4 weeks of age or older admitted for in-patient care were eligible for enrollment. After informed consent had been obtained, a nasal swab was collected within 48 hours of admission and comprehensive patient characteristics were entered into a standardized questionnaire.

Our study was powered to detect an MRSA colonization rate of 1% and this estimate was based on limited previous experience from an area in Switzerland.[12] Taking average numbers of yearly patient admissions at the 9 study sites into account, we aimed for approximately proportional enrollment of patients with 200 patients each in Basel, Bern, Geneva, Lausanne, and Zurich; 150 patients each in Aarau, Lucerne, and St. Gallen; and 50 patients in Bellinzona. The study was approved by each local ethical committee.

One cotton swab was used per patient for both nostrils, placed into the transport medium M40 Transystem (Copan, Brescia, Italy), and was sent overnight to the central bacteriological laboratory in Basel, except on weekends. For culture of *S. aureus*, a chromogenic medium (MRSA ID agar, bioMérieux, Marcy l'Etoile, France), and a selective enrichment broth (brain heart infusion broth with 6% NaCl, Biomedics, Madrid, Spain) were inoculated. After incubation overnight, the broth was subcultured onto another chromogenic agar for *S. aureus* (Chromagar *S. aureus*, Hy Laboratories, Rehovot, Israel). Identification of *S. aureus* was based on various traits such as typical growth on a chromogenic medium and detection of

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Journal of Clinical Virology 39 (2007) 22–26

JOURNAL OF CLINICAL VIROLOGY

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Direct detection of human herpesvirus 6 DNA in serum by the loop-mediated isothermal amplification method

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Received 29 August 2006; received in revised form 1 February 2007; accepted 1 February 2007

Abstract

Background: A more rapid and easier method is needed for monitoring human herpesvirus 6 (HHV-6) infections. The loop-mediated isothermal amplification method (LAMP) can detect viral DNA with high specificity, efficiency, and speed under isothermal conditions. LAMP requires only simple equipment that is available in hospital laboratories.
Objectives: We evaluated LAMP as a means of detecting HHV-6 DNA directly from patients' sera.
Results: The sensitivity of the HHV-6 LAMP protocol without heat denaturation was 1000 copies/tube; with heat denaturation 10 copies/tube were detected. Three hundred serum samples from children with fever were analyzed. Using HHV-6 isolation as a definition of HHV-6 infection, the sensitivity, specificity, positive predictive value, and negative predictive value of the HHV-6 LAMP method without DNA extraction were 95.5%, 95.2%, 94.0%, and 96.4%, respectively.
Conclusion: Direct detection of HHV-6 DNA in serum with a modified HHV-6 LAMP could be used for rapid diagnosis of exanthem subitum (ES).

Keywords: HHV-6; LAMP; Serum; Exanthem subitum

1. Introduction

Exanthem subitum (ES) is common febrile illness in infants caused by primary human herpesvirus 6 (HHV-6) B infection (Yamanishi et al., 1988; Asano et al., 1989). Although the disease usually is benign and self-limited (Asano et al., 1994), HHV-6 rarely can cause severe complications, such as encephalitis (Asano et al., 1992; Suga et al., 1993), fulminant hepatitis (Asano et al., 1990), hemophagocytic syndrome (Huang et al., 1990), and myocarditis (Yoshikawa et al., 2001a). As parents' anxiety about febrile infants is great, most patients with ES in Japan visit outpatient clinics. The clinical diagnosis is difficult before appearance of the skin rash, since high fever alone is characteristic of the acute phase of the disease (Asano et al., 1994). Therefore, a rapid laboratory method is needed to diagnose HHV-6 infection during the febrile period of ES. Additionally, reactivation of the virus can manifest in several ways, such as acute graft-versus-host-disease-like illness (Yoshikawa et al., 1991, 2001b, 2002), encephalitis (Drobyski et al., 1994; Singh and Paterson, 2000), bone marrow suppression (Drobyski et al., 1993; Carrigan and Knox, 1994; Ljungman et al., 2000), and interstitial pneumonitis (Carrigan et al., 1991; Cone et al., 1993) in organ transplant recipients. Thus, a rapid diagnostic procedure would be valuable for management of organ transplant recipients.

Abbreviations: HHV-6, human herpesvirus 6; LAMP, loop-mediated isothermal amplification; PBMCs, peripheral blood mononuclear cell; PCR, polymerase chain reaction

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doi:10.1016/j.jcv.2007.02.001

To date rapid diagnosis of HHV-6 infection has proven difficult. Isolation of HHV-6 requires co-cultivation with pre-activated cord blood mononuclear cells, which is difficult to perform in commercial laboratories. Moreover, both viral isolation and serological testing require substantial amounts of time to obtain final results. Although we established the antigenemia assay for HHV-6 immediate early antigen in peripheral blood mononuclear cells (PBMCs) (Nishimura et al., 2005), it is not practical for routine use in small hospital laboratories because of its complexity. Rapid diagnosis using polymerase chain reaction (PCR), in particular real-time PCR, may eventually become a valuable tool for monitoring of active viral infection (Ihira et al., 2002; Sashihara et al., 2002), but it is not a common procedure in small hospital laboratories that lack the required thermal cycler. As most ES patients visit private outpatient clinics or small hospitals, cheap and easy diagnostic procedures for ES would be desirable.

Notomi et al. (2000) reported a novel nucleic acid amplification method, termed loop-mediated isothermal amplification (LAMP) that amplifies DNA with high specificity, efficiency, and speed under isothermal conditions. The most significant advantage of the LAMP method is the ability to amplify specific sequences of DNA under isothermal conditions between 63 °C and 65 °C. As a result, it requires only simple and inexpensive equipment available in typical hospital laboratories. We developed the LAMP method for detection of six human herpesviruses (Ihira et al., 2004; Yoshikawa et al., 2004; Okamoto et al., 2004; Enomoto et al., 2005; Kimura et al., 2005; Sugiyama et al., 2005; Suzuki et al., 2006). Furthermore, we found that herpes simplex virus DNA could be detected directly in swab samples by the LAMP method without DNA extraction (Enomoto et al., 2005). Recently, we utilized LAMP after extraction of serum to specifically amplify HHV-6 DNA with high efficiency in order to diagnose active HHV-6 infection (Ihira et al., 2004). We describe below the application of the LAMP method for rapid diagnosis of HHV-6 infection by detecting HHV-6 DNA in serum without DNA extraction.

2. Materials and methods

2.1. Study design and study subjects

In order to detect HHV-6 DNA in serum directly, initial validation analysis was carried out as follows. The effect of a heat denaturation (96 °C for 30 s) step in the HHV-6 LAMP reaction on the lower limit of detection was evaluated by using serial dilutions of the pGEMH6 S12 plasmid in control serum that did not contain HHV-6 DNA. Additionally, in order to determine if serum was inhibitory in the LAMP reaction, serial dilutions of the pGEMH6 S12 plasmid in sterilized water and the control serum were used to determine the lower limit of detection.

A total of 300 febrile children (157 male and 143 female, 11.6 ± 8.7 months), who attended an outpatient clinic in our university hospital or Showa hospital, were enrolled into the study. Informed consent was obtained from the parents of all children. Four milliliters of blood (2 ml EDTA-treated and 2 ml clotted) were collected during the acute phase (febrile period) of the illness.

2.2. Isolation of HHV-6

The procedures for isolation and identification of HHV-6 have been described (Asano et al., 1989).

2.3. LAMP

Primers for HHV-6 LAMP (H6U31BIP; H6U31FIP; H6U31B3; H6U31F3) were designed for the HHV-6 B U31 gene using Primer Explorer V Soft Ware (Ihira et al., 2004). Since additional loop primers increase the amplification efficiency (Nagamine et al., 2002), H6U31LPB and H6U31LPF were also synthesized. The LAMP reaction was carried out using the Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tokyo, Japan) according to the manufacturer's instruction. LAMP was carried out with 25 μl of a mixture containing 2.4 μM H6U31FIP and H6U31BIP primers, 0.4 μM of each outer primer (H6U31F3 and H6U31B3), 1.2 μM of each loop primer (H6U31LPF and H6U31LPB), 20 mM Tris–HCl [pH 8.8], 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.8 M betaine, 1.4 mM of each d-NTPs, 0.1% Tween20, 8U of *Bst* DNA polymerase, and 5 μl of template DNA. The mixture was incubated at 63 °C for 60 min, and increased turbidity was monitored by using LA-200 (Teramecs Co., Ltd., Kyoto, Japan) (Mori et al., 2002). In order to increase the sensitivity of the method, a heat denaturation step (96 °C for 30 s) was added as follows: the mixture without *Bst* DNA polymerase containing template DNA (patient's serum) was incubated at 96 °C for 30 s, after which 1 μl (8U) of *Bst* DNA polymerase was added, and this mixture incubated at 63 °C for 60 min. The LAMP products were detected turbidometrically using LA-200. The cutoff value for discrimination between positive and negative samples was 0.1, which was determined by analyzing five virus-negative samples. During the initial analysis of the effects of heat denaturation and inhibitory effects of serum, agarose gel electrophoresis was also used to detect LAMP products. Six microliters of LAMP products were subjected to electrophoresis on a 1.5% agarose gel. After staining with ethidium bromide, the products were detected using UV light.

3. Results

In order to evaluate the effect of heat denaturation on assay sensitivity, the lower detection limits were compared with and without a heat denaturation step. The HHV-6 LAMP protocol with heat denaturation was 100 times more sensitive

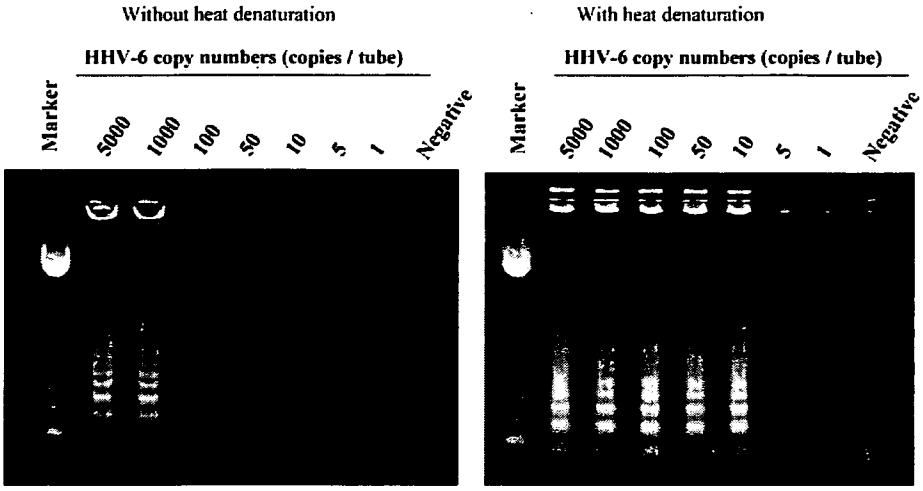

Fig. 1. Evaluation of heat denaturation (96 °C for 30 s) on the sensitivity of HHV-6 LAMP. Serial dilutions of pGEMH6S12 plasmid DNA in control serum with or without heat denaturation were amplified by the LAMP.

(10 copies/tube) than when heat denaturation was omitted (Fig. 1).

Direct detection of HHV-6 DNA in patients' serum by the LAMP method should provide a more rapid and easier diagnostic procedure. In order to evaluate the sensitivity of such an assay, potential inhibitory effects of serum were explored. In reconstruction experiments the lower limit of detection of HHV-6 in sterilized water was 5 copies/tube, while in serum 10 copies/tube were detected, using either the turbidometric or agarose gel electrophoresis assays (Fig. 2). Furthermore, threshold time (time to reach the threshold level of turbidity of 0.1) of each plasmid dilution in sterile water was faster than when serum was present (Fig. 2).

The results of viral isolation and direct serum HHV-6 LAMP were compared to assess the reliability of the direct LAMP method for rapid diagnosis of primary HHV-6 infection. HHV-6 was isolated from 132 (44.0%) of the 300 PBMCs samples collected (Table 1). HHV-6 DNA was detected in 126 (95.5%) of the 132 serum samples collected from the subjects with HHV-6 viremia, while HHV-6 DNA was detected in 8 (4.8%) of the 168 serum samples without HHV-6 viremia. Thus, if virus isolation was defined as standard for active virus infection, sensitivity, specificity, positive predictive value, and negative predictive value of serum HHV-6 LAMP were 95.5%, 95.2%, 94.0%, and 96.4%, respectively. HHV-6 DNA became positive by LAMP in four of the six false negative serum samples after DNA extraction from the serum.

Table 1
Comparison between viral isolation and serum LAMP was performed to evaluate the reliability of direct HHV-6 LAMP method as the diagnostic method for primary HHV-6 infection

Results of HHV-6 LAMP	Isolation of HHV-6	
	Positive	Negative
+	126	8
−	6	160

Sensitivity: 95.5%, positive predictive value: 94.0%, specificity: 95.2%, negative predictive value were: 96.4%.

4. Discussion

It has been demonstrated that the sensitivity of direct amplification of HSV DNA by the LAMP method is slightly less sensitive than when it is applied to DNA first extracted from the clinical sample (Enomoto et al., 2005). Moreover, serum has a slight inhibitory effect on the LAMP reaction (Fig. 2). Thus, it was expected that an increase in assay sensitivity would be required to detect viral DNA in serum without DNA extraction. The addition of a heat denaturation step greatly increases the sensitivity of the LAMP method (Poon et al., 2006), and we found that heat denaturation resulted in assay sensitivity up to 10 copies/tube, as measured by agarose gel electrophoresis (Fig. 1). We concluded that the modified protocol with a heat denaturation step (lower detection limit; 10 copies/tube) should be sufficient for clinical use.

The LAMP method with an initial heat denaturation step was sufficiently sensitive to detect HHV-6 directly from sera from febrile children. The effect of inhibitors in sera was negligible. Furthermore, the use of a turbidometric assay permitted the quick identification HHV-6 DNA-positive sera using equipment available in most hospital laboratories. The method also had a high sensitivity and specificity for the diagnosis of primary HHV-6 infection. As a matter of fact, turbidity of all of the positive samples reached at threshold level (0.1) within 45 min (data not shown) and the turbidity curve of the lower detection limit (10 copies/tube) reached its threshold after 39 min of LAMP reaction (Fig. 2 A). Since this protocol does not require DNA extraction, the final result is obtainable within 60 min after collecting the clinical specimens.

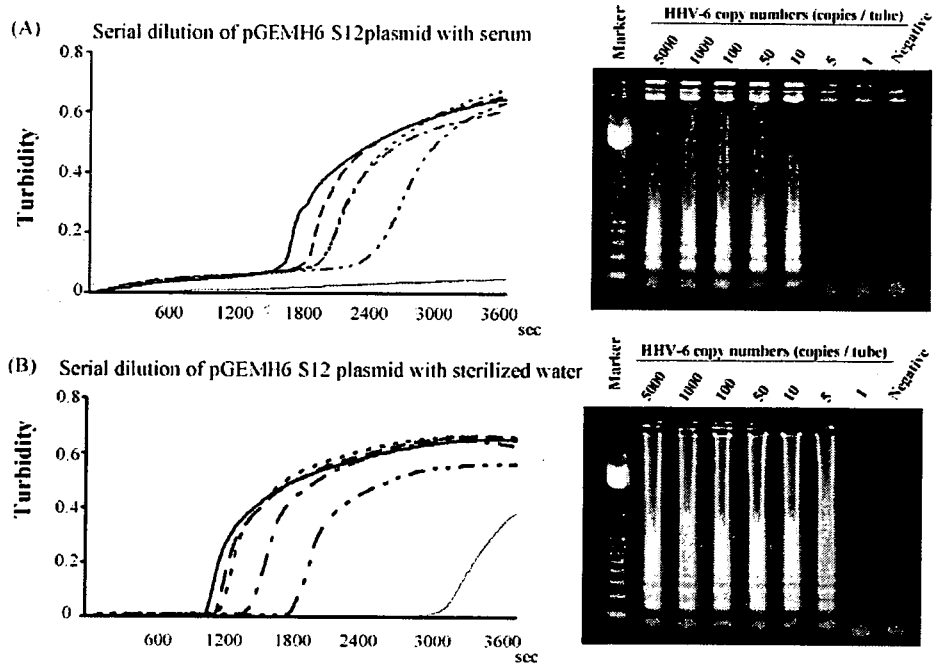

Fig. 2. Evaluation of the inhibitory effect of serum in the LAMP reaction. Serial dilutions of pGEMH6S12 plasmid DNA in either control serum (A) or sterile water (B) were amplified after heat denaturation. The LAMP products were detected by agarose gel electrophoresis and LA-200 (as turbidity). M, marker; Neg., negative; ▬▬, 5000 copies/tube; ▬ ▬, 1000 copies/tube; ..., 100 copies/tube; ▬ ▪ ▬ 50 copies/tube; ▬ ▪ ▪ 10 copies/tube; —, 5 copies/tube; - - -, 1 copies/tube; ▬..▬..▬, without plasmid DNA.

Some of the false negative samples became positive after DNA extraction. It is possible that these samples contained small amounts of viral DNA that were below the detection limit of the direct HHV-6 LAMP method. Since virus isolation was defined as the marker for viral infection in this study, it is possible that some of the "false positive" results are incorrectly labeled. Serological analysis in combination with viral isolation would be necessary to determine the precise reliability of this method in future experiment.

To our knowledge, this is the first report to demonstrate direct amplification of viral DNA from serum samples without DNA extraction by this molecular biological method. The rapidity of the method permits its use for diagnosis of ES in acutely ill children. It could also be used for management of transplant recipients and could be used for rapid detection of the other pathogens.

Acknowledgements

We thank Takeda Chemical Industries, Ltd., Osaka, Japan, for supplying recombinant human interleukin-2 for cultivation of lymphocytes and gratefully acknowledge Eiken Chemical for their contribution to carry out this work. We also thank Mrs. Akiko Yoshikawa and Ms. Maki Sawamura for technical assistance.

This work was supported in part by a Grant-in-Aid for the 21st Century COE Program of Medicine of Fujita Health University and the Open Research Center of Fujita Health University, from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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Familial amyloid polyneuropathy (Finnish type) in a Japanese family: Clinical features and immunocytochemical studies

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Received 1 March 2006; received in revised form 31 August 2006; accepted 5 September 2006
Available online 13 November 2006

Abstract

Familial amyloid polyneuropathy (FAP: type IV), known as familial amyloidosis of the Finnish type (FAF), is very rare and reported only in a few countries.

The *gelsolin* mutation G654A is most frequent causative gene in FAF family. The clinical phenotype of FAF possesses several neurological characteristics with multiple cranial nerve signs, in addition to a peculiar exanthema of "lichen amyloidosus" and pendulous skin "cutis laxa", and the carpal tunnel syndrome.

We report a new Japanese FAF family presenting bilateral atrophies and fasciculations of the facial muscles and tongue. The patients in our family presented with skin changes as "lichen amyloidosus" and "cutis laxa". In this FAF family, lichen amyloidosus appeared under sunlight and high temperatures in the summer season every year. Two patients in our family presented with common clinical features of FAF, except for the above laboratory results. Including previous cases and our family, this clinical phenotype is similar to the gelsolin gene mutation (G654A) in FAF family members.

Keywords: Familial amyloid polyneuropathy; Finnish type; Gelsolin mutation (G654A); Corneal lattice dystrophy; Cutis laxa; Lichen amyloidosus

1. Introduction

Familial amyloidosis of the Finnish type (FAF), known as familial amyloid polyneuropathy (FAP) type IV, occurs in autosomal dominant inheritance and was first described by Meretoja in 1969 [1]. FAF is rarely reported in only a few countries including Finland, Denmark, Holland, the USA, Portugal and Japan [2]. The main clinical features are peripheral polyneuropathies including multiple cranial nerves, corneal dystrophy and skin changes (cutis laxia and lichen amyloidosus) [3,4], while dysphagia and dysarthria may occur due to bulbar palsy [1,3]. The autonomic nervous systems of FAF patients are slightly affected [5]. Mild, predominantly sensory, peripheral neuropathies, as well as carpal tunnel syndrome, have also been described [6]. Amyloid deposition is observed in the skin, cornea, vascular walls, and peritoneum, especially in the facial nerves [7–9]. Biochemical studies have indicated that amyloid fibrils in FAF are related to gelsolin, an actin-modulating protein with a single amino acid substitution at position 187, resulting in asparagine for aspartic acid [10]. The gelsolin gene is located on chromosome 9q34 [11] and molecular analysis has demonstrated a G-to-A substitution at nucleotide 654

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doi:10.1016/j.jns.2006.09.022

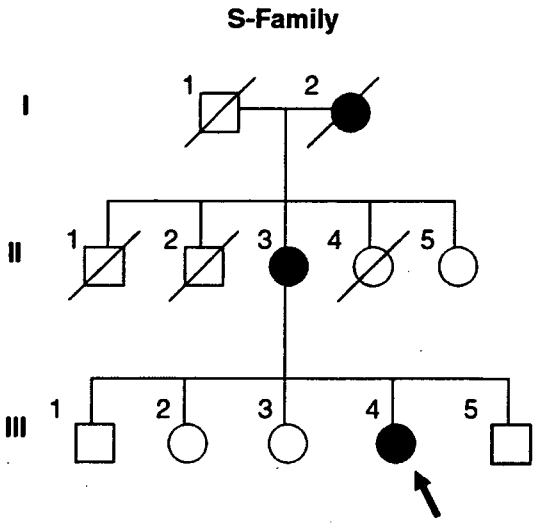

Fig. 1. Pedigree of FAF (S-family). Male family members are represented by squares; females by circles; deceased members by oblique lines; affected members by solid symbols. The proband is indicated by an arrow (III-4). I-2 was said to suffer from bilateral facial palsies, suspected to be FAF.

(G654A) in the gelsolin gene [12]. Including the first case of Japanese FAF family reported by Sunada et al. [13], a few FAF families have been published in Japan [14–16]. Here, we report another Japanese FAF family presenting with neurological symptoms of VII and XII cranial nerves and lichen amyloidosus skin lesions.

2. Case presentation

At least two patients showed clinical symptoms of FAF in three generations of our family, including one suspected FAF subject. They have lived in Gunma prefecture, Japan, for many generations and have no relationship with Finnish ancestors or other Japanese FAF families (Fig. 1). The first symptom at the onset of the disease in the proband (III-4, 56 years old at present) of our family was bilateral peripheral facial nerve palsies at 51 years old. Soon later, she presented with fasciculations (lower eye lid, cheek, perioral and mandibullar areas) dominantly on the right side. At the examination, she showed bilateral peripheral facial nerve palsies (Fig. 2 A), tongue atrophy in the central area around macroglossia in the periphery (Fig. 2 B) and fasciculations of the tongue. She also showed generalized hyporeflexia, without muscular weakness or atrophy, ataxia and sensory disturbances. Lichen amyloidosus skin changes appeared in the bilateral forearms and hands, and pendulous skin (cutis

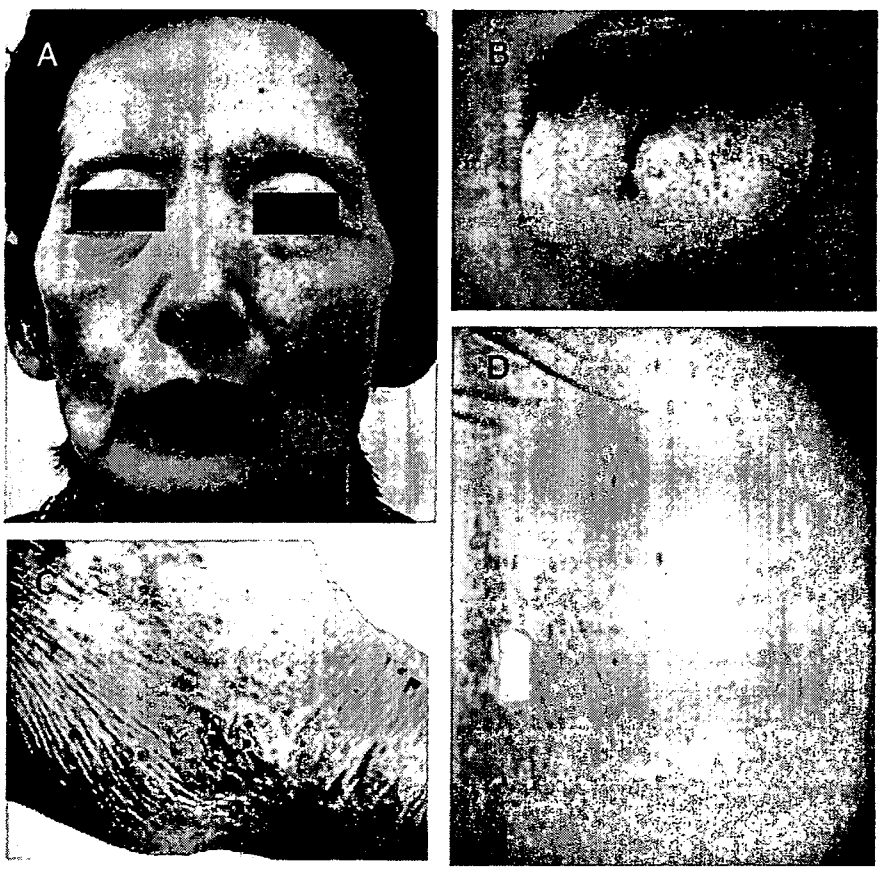

Fig. 2. The proband of this FAF family. A. Appearance of the proband (III-4). Bilateral facial palsies in the forehead, and skin relaxation (cutis laxa) of the forehead, upper lids, cheek and angle of the mouth with right dominance. B. Tongue atrophy, especially in the central area. C. Lichen amyloidosus and cutis laxa at the right hand joint and dorsum of hand. D. Lattice corneal dystrophy shown by slit lamp examination.

Table 1
Median nerve examination (right)

	TL/6 cm	CV	Amp
Motor	3.28 ms palm	54.3 m/s palm–wrist	7.9 mV wrist–elbow
Sensory	33.0 m/s	37.1 m/s	55.6 m/s

The nerve conduction velocity of the right median sensory nerve was delayed at the palm, and between the palm and elbow. Amplitude of the potential is slightly low.

laxa) was observed in the bilateral forearms, hands and face (Fig. 2 C). Slit lamp examination revealed scattered multiple streaks on the cornea (corneal lattice dystrophy (type II)) (Fig. 2 D), which was compatible with a characteristic feature of FAF. No abnormal findings were observed on MRI of the brain and spinal cord by either plain imaging or Gadolinium (Gd) enhancement.

The first recognized symptom of the proband's mother (II-3, 86 years old at present) at the onset was also bilateral facial palsies at 50 years old. She had the same clinical symptoms as the proband (III-4), which were 1) bilateral peripheral VII and XII nerves cranial nerve palsies with fasciculations, 2) corneal lattice dystrophy in bilateral eyes, 3) lichen amyloidosus and pendulous skin (cutis laxa) with severe itching, skin rash and pigmentation in the bilateral forearms, hands, neck and feet. The lichen amyloidosus in the bilateral hands and posterior cervical region appeared every summer, the same as in the proband. The grandmother (I-2) of the proband was said to have had similar neurological symptoms to the two patients (III-4 and II-3) by the relatives in our family. None of the family members, including the FAF patients, have suffered from dementia.

3. Laboratory examinations

Electrophysiological examination of the right median nerve of the proband (III-4) showed delayed sensory conduction velocity (Table 1). These results suggested that subclinical carpal tunnel syndrome or subclinical peripheral neuropathies might exist, although she had no sensory complaints.

4. Histology and immunocytochemical examinations

After we have had informed consent from the proband (III-4),we biopsied a piece of skin tissue from the right biceps brachii. The sample was fixed in 4% paraformaldehyde and embedded in paraffin. The method of immunocytochemistry

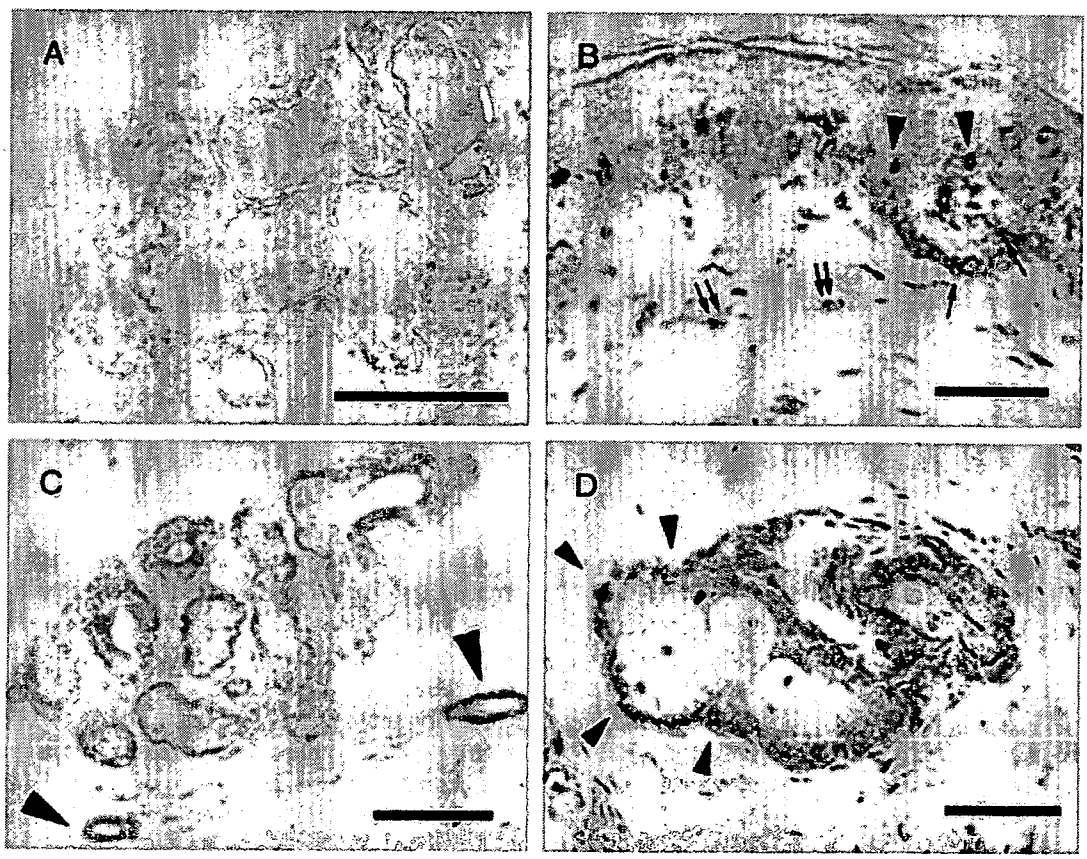

Fig. 3. Histological and immunocytochemical findings of skin in the proband. A. Congophilic amyloid deposits were observed at the outline of eccrine gland cells with polarizing microscopy. B. Immunocytochemistry using an anti-gelsolin monoclonal antibody showed positive stainings in several cells of the epidermis ( ), cells in the stratum basale of epidermis ( ), and several cells in the dermis ( ). C. Gelsolin-immunoreactive structures were observed at the outline of the eccrine glands and in vascular walls ( ). D. Gelsolin-immunoreactive structures are observed at the outline of the tissues of sebaceous glands ( ). Scale bar=50 m (A–D).

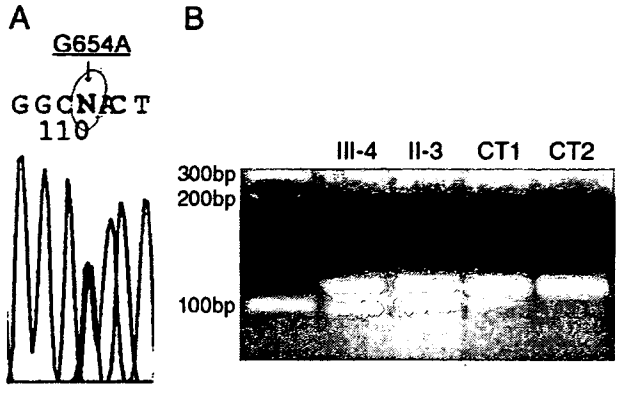

Fig. 4. Molecular analysis. A. Sequencing of the PCR product spanning codon 187 of genomic gelsolin DNA. The proband (III-4) showed heterozygotes by showing "N" for both normal and mutant alleles, guanine to adenine at the first base of codon 187 (arrow) in the gelsolin gene. B. Restriction enzyme assay of the PCR product using a modified primer in 4% agarose gel. PCR products of II-3 and III-4 were digested to 116 bp and 90 bp by the MfeI enzyme, while PCR products of the control subjects were not digested so that only 116 bp appeared (CT represented control subject).

using anti-gelsolin monoclonal antibody (G4896, 1:500, Sigma) followed previous methods [17]. Congo red staining demonstrated yellow–green birefringence of the deposits at the outlines of eccrine sweat glands in polarizing microscopy (Fig. 3 A). Immunocytochemistry using an anti-gelsolin antibody showed positive stainings in several cells of the epidermis, stratum basale of the epidermis, and dermis (Fig. 3 B). The outline of the eccrine sweat glands presented gelsolin-positive immunoreactivity and vascular walls (Fig. 3. C:). Immunoreactive structures were also observed at the outline of sebaceous glands (Fig. 3 D). Immunocytochemical examination by the same G4896 antibody revealed that the skin tissues from two non-FAF subjects showed negative staining (data not shown).

5. Mutation analysis

After obtaining informed consent for this genetic test, we purified genomic DNA from lymphocytes in the peripheral blood of the two subjects (III-4 and II-3). Purified genomic DNA was sequenced according to a previous method [18,19]. A DNA fragment (116 bp) containing codon 187 was amplified by PCR using the primers suggested by Levy et al [12]. The primer sequences were as follows: primer A (forward), 5 -TGGTGGTGCAGAGACTCTTCC-3 ; primer B (reverse), 5 -GTTGCCCAGGTCCAGGAT-3 . One microgram of genomic DNA isolated from leukocytes was mixed with 0.5 g of each primer, followed by amplification through 30 cycles under the following conditions: 94 °C for 30 s for denaturation, 60 °C for 30 s for annealing, and 72 °C for 30 s for elongation. The PCR product was excised from 4% Nusieve (FMC Bioproducts, Rockland, ME) agarose gel, purified, and then subjected to direct sequencing using an ABI PRISM® 3100 Genetic analyzer DNA sequencer (Applied Biosystems, Foster City, CA, USA).

To confirm the missense mutation, we used modified PCR for restriction analysis as below [13]. Genomic DNA extracted from leukocytes was subjected to PCR with the primer A and modified primer B (5 -GTTGCCCAGGTCCAGGAT-GAAGCAAT-3). Primer B has a mismatched adenine residue at the second position from the 3 end and generates an Mfe I recognition site only if the guanine to adenine substitution recognizes nucleotide 654 of the gelsolin gene. The missense mutation of the gelsolin gene was confirmed as G654A (G-to-A substitution at nucleotide 654), an amino acid substituted coding 187 resulting in a change from aspartic acid to asparagine, which detected mutant and wild type gelsolin as a heterozygote (II-3 and III-4) (Fig. 4. A). Mfe I (New England Biolaboratory) digestion of PCR product flanking at nucleotide 654 showed 126 bp and 90 bp in II-3 and III-4, while no such missense mutation was detected in 100 normal control alleles (Fig. 4. B).

6. Discussion

The clinical phenotype of FAF presented wide ranges of multiple cranial nerve palsies, mainly VII nerve, additionally V, IX, X, XI and XII nerves, rarely III, IV and VI nerves, with corneal lattice dystrophy and lichen amyloidosus skin changes and cutis laxa, occasionally with carpal tunnel syndrome and orthostatic hypotension [1–6].

We reported one FAF Japanese family with neurological deficits including only two cranial neuropathies (VII, XII), showing lichen amyloidosus with seasonal periodicity which deteriorated under strong sunlight and high temperature every summer. Kiuru-Enari et al. first reported that amyloid-positive structures for the anti-gelsolin antibody were detected in the vascular walls and connective interstitial tissues in both skin and muscles [9]. A skin biopsy of the proband demonstrated that gelsolin-positive structures were observed in several cells of epidermis, dermis, stratum basale, and around the eccrine and sebaceous glands cells in dermis. Our study also showed that deposition of gelsolin in vascular walls of skin tissues was similar to previous reports of FAF ceases [1–6,13–16]. The right median nerve of the proband (III-4) showed delayed velocity of sensory conduction, suggesting that subclinical carpal tunnel syndrome might exist or subclinical peripheral neuropathies might occur due to mild degeneration of axons and the myelin of the peripheral nerves [6]. Only four FAF families have been reported in Japan to date [13–16], and there is no report from any other Asian countries.

In previous Japanese cases of FAF, Sunada et al. described five cranial nerves deficits of V, VII, IX, X and XII [13]. Makishita et al. mentioned nine cranial nerves deficits of III, IV, VI, V, VII, IX, X, XI and XII [14]. Ishiguchi et al. reported four cranial nerves deficits of VII, IX, X and XII [15]. There were clinical divergences even in Japanese FAF cases. Compared with previous FAF cases in Japan, both patients in our family showed only two cranial nerves deficits, especially the proband's mother still remain the same cranial

nerves symptoms even over 80 years old. In the points of mild cranial nerves deficits and seasonal skin exanthema common to two patients, our FAF family possesses very peculiar clinical characteristics in not only Japan but all over the world.

Including previous reports, the intrafamilial clinical findings are similar to the gelsolin gene mutation (G654A) in FAF. The cause of divergence in clinical phenotypes among different independent FAF families bearing the same gelsolin mutation should be clarified, especially to elucidate unknown modifier gene(s) for the clinical divergence in FAF.

Acknowledgement

We thank Dr. Yoichi Nakazato for the technical supports.

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Brain & Development 29 (2007) 447–449

BRAIN & DEVELOPMENT
Official Journal of the Japanese Society of Child Neurology

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Case report

West syndrome associated with mosaic Down syndrome

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Received 13 July 2006; received in revised form 14 December 2006; accepted 25 December 2006

Abstract

We report a girl with West syndrome associated with mosaic Down syndrome. She had repetitive tonic spasms at 6 months and an electroencephalography (EEG) showed hypsarrhythmia. Her facial appearance was normal and she had no minor anomalies. Her karyotype was mosaic(46,XX/47,XX,+21). Adrenocorticotropin (ACTH) therapy was effective, and her developmental quotient was 76 at 17 months. This report re-emphasize that chromosomal analysis is recommended for epileptic patients with infantile onset when the cause is unclear.

Keywords: Mosaic Down syndrome; West syndrome; Chromosome

1. Introduction

Down syndrome is a genetic disturbance that is most frequently associated with intellectual disability with an estimated frequency rate of one in 650–1000 births. Although, the majority of people with Down syndrome have full chromosome 21 trisomy, it is estimated that 2.4% of patients with Down syndrome show mosaicism, both normal and trisomic cell lines [1]. It is well-known that Down syndrome is often complicated with West syndrome [2]. However, there has not previously been a case report of West syndrome associated with mosaic Down syndrome. We here report a case of mosaic Down syndrome complicated with West syndrome.

2. Case report

A 6-month-old girl developed repetitive tonic spasms in clusters, and was referred to the hospital. She was born at 41 weeks and 4 days of gestation with an emergency cesarean section due to a decrease in fetal heart rate. Apgar scores at 1 and 5 min were 8 and 9, respectively. She was the first child of healthy unrelated parents with no remarkable family history. Her developmental milestones were normal at 3 months. She then deteriorated from 4 months showing decreased smiling and disability of head control. She had tonic spasms once or twice a day at 5 months, and then the spasms became more frequent, occurring in series. Electroencephalography (EEG) showed hypsarrhythmia. She was diagnosed as having West syndrome, and treated by vitamin B_6 (30 mg/kg/day) for 1 week. Thereafter, she was admitted to our hospital at the age of 6 months for adrenocorticotrophic hormone (ACTH) therapy because vitamin B_6 was not effective. Her facial appearance was normal (Fig. 1). There were no minor anomalies suggesting Down syndrome, including short neck with skin folds, brachydactyly, clinodactyly, simian crease, small middle phalanx of the second and fifth digits, and wide space and furrow between toes 1 and 2. Her developmental quotient was 47. She could not control her head or follow a moving object. Her muscle tone and deep tendon reflex were normal. The following lab-

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doi:10.1016/j.braindev.2006.12.006

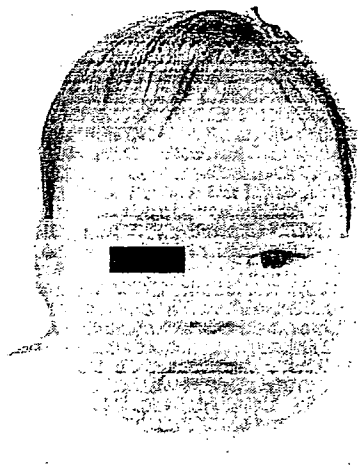

Fig. 1. Facial appearance of the patient.

oratory tests were normal: complete blood counts, serum chemistry results, and serum electrolytes. Computed tomography and magnetic resonance imaging of the brain did not demonstrate any abnormalities. Chromosomal analysis demonstrated 46,XX in 28 cells, and 47,XX,+21, in two of 30 cells. We diagnosed her as having mosaic Down syndrome (46,XX/47,XX,+21). The EEG demonstrated hypsarrhythmia on admission (Fig. 2a). She was treated with ACTH at a dosage of 0.0125 mg/kg daily for 2 weeks and gradual tapering in subsequent 2 weeks. Her spasms disappeared and her psychomotor function developed. The EEG on day 24 of admission was normal (Fig. 2b). Then, she was treated with valproate, and her developmental quotient was 76 at 17 months (Fig. 3).

3. Discussion

Mosaic Down syndrome demonstrates two karyotypes, 46 normal karyotype and 47 karyotype specific for Down syndrome, and clinical manifestations are dependent on the proportion of trisomy cells, which renders clinical diagnosis difficult [3]. Mosaic Down syndrome accounts for 2.4% of Down syndrome.[1] It has been reported that 90% and 100% of standard and translocation Down syndrome, respectively, could be diagnosed from clinical symptoms, but only 37.5% of mosaic Down syndrome could be clinically diagnosed [4]. We could diagnose our patient as having Down syndrome by chromosomal analysis, but not clinical symptoms. Intelligence in patients with mosaic Down syndrome is related to the ratio of trisomy cells. The previous report demonstrated that the average intellectual quotient of mosaic Down syndrome with 50% or higher of normal cell proportions was 57, while that of those with less than 50% [5]. Because she had 93% normal cell proportions and West syndrome was well controlled quickly, the developmental quotient in this case was 76 at 17 months.

With regard to the etiology in 77 patients with the prenatal symptomatic West syndrome, Ohtahara et al. reported that brain malformation was the most frequent abnormality found in 31 cases, followed by tuberous

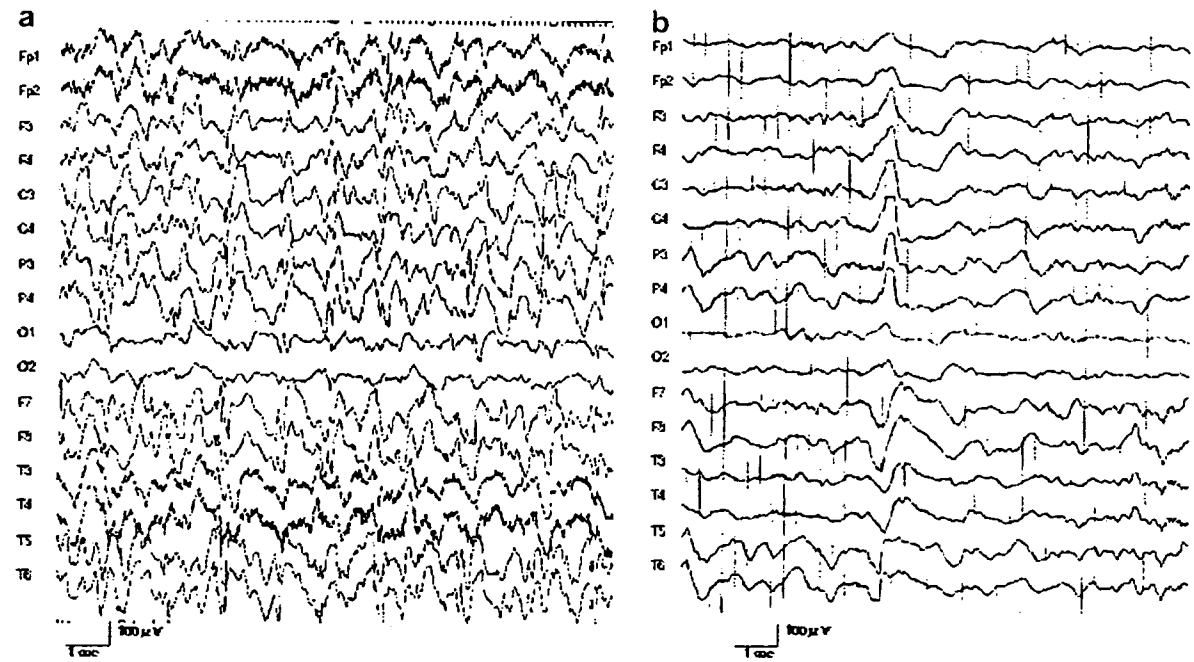

Fig. 2. Interictal sleep EEG of the patient. Hypsarrhythmia was presented on admission (a). Hypsarrhythmia was disappeared after ACTH therapy (b).

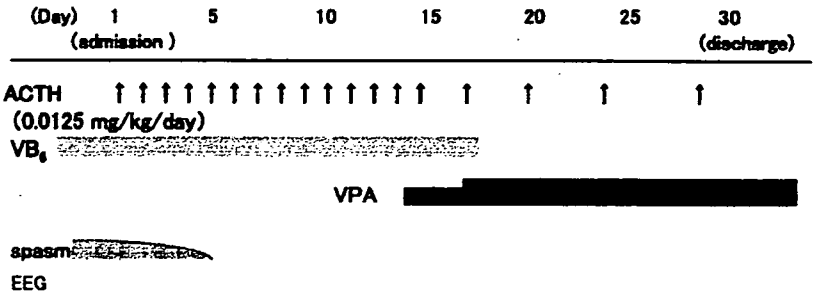

Fig. 3. Clinical course of the patient. The day of admission was defined as day 1. VB_6, vitamin B_6; VPA, valproic acid.

sclerosis in 23 and chromosomal abnormality in 10. As to chromosomal abnormality, Down syndrome was found most frequently in six of 10 [6]. It has been reported that 6.4% of 737 patients with Down syndrome had epilepsy, and 12.8% of epileptic patients with Down syndrome had West syndrome [7]. In addition, it has been reported that 8.1% of 405 patients with Down syndrome had epilepsy in childhood, and 18% of the epileptic patients were diagnosed as having West syndrome [8]. West syndrome is a frequently observed form of epilepsy in Down syndrome [9]. However, there has not been any previous case report describing West syndrome with mosaic Down syndrome.

A majority of lesions responsible for epilepsy at infantile onset are attributable to prenatal causes [10]. Chromosomal abnormality is one of the causes. The diagnosis of chromosomal abnormality is not as easy as it initially appears in some patients. We encountered an infant with mosaic Down syndrome who had a completely normal facial appearance. This case re-emphasizes that chromosomal analysis should be performed for the epileptic patients with infantile onset when the cause is unclear.

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European Journal of Neurology 2007 doi:10.1111/j.1468-1331.2007.01978.x

SHORT COMMUNICATION

Clinical features of patients with myasthenia gravis associated with autoimmune diseases

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Keywords:
autoimmune disease,
Graves' disease,
Hashimoto's thyroiditis,
myasthenia gravis

Received 3 August 2007
Accepted 6 September 2007

We investigated the incidence and clinical features of patients with myasthenia gravis (MG) associated with autoimmune diseases. Associated autoimmune diseases were found in 28 of 142 consecutive Japanese MG patients (19.7%), amongst which Graves' disease (7.7%) and Hashimoto's thyroiditis (4.2%) were predominant. The clinical features of MG patients with Graves' disease were different from those of MG patients without autoimmune diseases in terms of age at onset of MG symptoms (35.5 ± 4.0 years and 49.0 ± 1.7 years; $P < 0.05$), positivity for the anti-acetylcholine receptor antibody (44.4% and 89.8%; $P < 0.05$), and association with thymic hyperplasia (72.7 and 17.9%; $P < 0.05$). The therapeutic outcome of MG patients with Graves' disease and that of those without autoimmune diseases were not significantly different. Further studies should be performed to investigate whether MG associated with Graves' disease is a distinct subtype of MG.

Introduction

Myasthenia gravis (MG) is an autoimmune disorder characterized by an impaired neuromuscular transmission caused by circulating anti-acetylcholine receptor antibody (AChR Ab) or other aetiologies [1]. The association of MG with other autoimmune diseases sometimes occurs coincidentally [2]. The rates of association in a Norwegian and Danish studies were 22.9% (11/48 patients) and 9.4% (20/212 patients) respectively [2,3]. The Danish study has also shown that autoimmune thyroid diseases (AITDs) such as Graves' disease and Hashimoto's thyroiditis are most frequently associated with MG [3]. In this study, we examined the incidence, clinical features, and thymic histology of Japanese patients with MG associated with other autoimmune diseases.

Patients and methods

We performed a retrospective analysis using medical charts to determine the incidence of autoimmune diseases amongst consecutive MG patients, who were admitted to our hospital between May 1986 and October 2005. Children with MG (< 10 years) were excluded from this study. The diagnosis of MG was made on the basis of results of clinical examinations, an edrophonium chloride test, and electrophysiological studies. The diagnoses of Graves' disease and Hashimoto's thyroiditis were made as described previously [4] and that of connective tissue diseases was made in accordance with the criteria of the American Rheumatism Association. Therapeutic outcome after extended thymectomy was evaluated at the end of the follow-up or in September 2006 using a modified method of evaluating MG outcome status [5]. Comparisons of the gender, positivity for AChR Ab, thymic histology, and therapeutic outcome were carried out using Fisher's exact test. The mean age at onset of MG symptoms for each group was subjected to unpaired Student *t*-test. Results were expressed as mean ± SEM.

Results

We examined 142 patients with MG (46 men and 96 women; age range, 10–84 years; mean age, 48.5 ± 1.5 years). AChR Ab titre was measured in all the patients at screening, although antibody titres to a muscle-specific kinase were not measured [1]. Thyroid functions including the serum levels of serum thyroxine, triiodothyronine and thyroid-stimulating hormone were also determined in all the patients. The titre of antinuclear and disease-specific autoantibodies was measured when necessary. Autoimmune diseases were found in 28 of the 142 patients with MG (19.7%), amongst which AITDs such as Graves' disease (11 patients; 7.7%) and Hashimoto's thyroiditis (six patients; 4.2%) were predominant. Other associated autoimmune diseases were as follows: rheumatoid arthritis ($n = 6$), systemic lupus erythematosus ($n = 2$), autoimmune thrombocytopenia ($n = 2$), and Sjögren's syndrome ($n = 1$).

We next investigated the clinical features of the MG patients with Graves' disease, and compared their clinical

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features with those of the MG patients without autoimmune diseases. The mean age at onset of MG symptoms in the patients with Graves' disease (35.5 ± 4.0 years) was younger than that in the patients without autoimmune diseases (49.0 ± 1.7 years) ($P < 0.05$). Female predominance was observed in both the MG patients with Graves' disease (81.8%) and in those without autoimmune diseases (64.9%), although there was no significant difference. The positivity for AChR Ab in the MG patients with Graves' disease (44.4%; 4/9 patients) was lower than that in the MG patients without autoimmune diseases (89.8%; 88/98 patients) ($P < 0.05$). The frequency of thymic hyperplasia in the MG patients with Graves' disease was much higher (72.7%; 8/11 patients) than that in the MG patients without autoimmune diseases (17.9%; 15/84 patients) ($P < 0.05$). The therapeutic outcome of the MG patients with Graves' disease was not significantly different from that of the MG patients without autoimmune diseases.

We also investigated the clinical features of the MG patients with Hashimoto's thyroiditis. The mean age at onset of MG symptoms in these patients (66.0 ± 2.2 years) was older than that in the MG patients without autoimmune diseases ($P < 0.05$). Thymic hyperplasia was not observed in this group. There was no significant difference in gender (female: 66.7%), the positivity for AChR Ab (83.3%; 5/6 patients), or therapeutic outcome between the MG patients with Hashimoto's thyroiditis and those without autoimmune diseases.

Discussion

We found that about 20% of Japanese MG patients in our hospital have associated autoimmune diseases and that AITDs were most predominant. These findings are similar to those of a previous study in Denmark [3]. We also found that the clinical features of MG patients with Graves' disease were an early onset of MG symptoms, female predominance, a low positivity for AChR Ab, and a high frequency of association with thymic hyperplasia. Although the positivity for AChR Ab in MG patients with Graves' disease has not been reported to date, a previous Japanese study demonstrated that an early age at onset of MG symptoms (mean age, 24.4 years) and female predominance (72.7%) are the clinical features of these patients [6]. Furthermore, the mean age at onset of MG symptoms in the patients with Graves' disease (35.5 ± 4.0 years) was younger than that in Japanese patients with MG (41.1 years) reported previously [7]. Thus, an early age at onset of MG symptoms might be a clinical feature of MG patients with Graves' disease. Note that MG patients with Graves' disease frequently exhibited thymic hyperplasia. There have been no reports describing thymic histology in MG patients with Graves' disease to date, although thymic hyperplasia has been shown to be more predominant than thymoma in MG patients with AITDs [4].

We also investigated the clinical features of MG patients with Hashimoto's thyroiditis. We found that the clinical features of these patients are similar to those of MG patients without autoimmune diseases except for the late age at onset of MG symptoms and the absence of association with thymic hyperplasia. Because it has been reported that the peak age range at onset in Japanese patients with Hashimoto's thyroiditis (40–49 years) was older than that in Japanese patients with Graves' disease (20–29 years) [8], the late age at onset of MG symptoms in MG patients with Hashimoto's thyroiditis might be reflected on the different mean ages at onset of the diseases. Additional studies should be performed to reveal the clinical features of MG patients with Hashimoto's thyroiditis.

The limitation of this study should be acknowledged. A P-value of 0.05, as are all the P-values in this study, is barely statistically significant. When the number of patients is not sufficient, as in this study, increasing or decreasing the number of patients by one might make the difference statistically significant or not. Additional studies are required to confirm our results.

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