

Retrospective Diagnosis of Congenital Cytomegalovirus Infection in Children with Autism Spectrum Disorder but No Other Major Neurologic Deficit

Running title: Congenital CMV infection and autism

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ABSTRACT

Aim: Congenital cytomegalovirus (CMV) infection can cause a variety of neurological deficits of delayed onset in infants who are asymptomatic at birth. The aim of this study was to investigate the prevalence of congenital CMV infection among children with autism spectrum disorder (ASD) in Nagasaki, Japan.

Methods: Twenty-nine children with ASD who were born in Nagasaki and had no other major neurological deficits were recruited. Two of the patients were excluded due to significant perinatal events. The remaining 27 children were investigated retrospectively for congenital CMV infection by analyzing dried blood spot samples or dried umbilical cords for CMV DNA using real-time PCR.

Results: CMV DNA was detected in two (7.4%) of the 27 children. Neither of the patients had perinatal histories suggestive of congenital CMV disease or other neurological deficits, including hearing impairment and epilepsy. The severity of their autistic disorders varied considerably.

Conclusions: The rate of congenital CMV infection in this study (two of 27 children with ASD), which was significantly ($p = 0.004$) higher than the incidence of congenital CMV infection in Nagasaki (0.31%, 10/3,230 live births), suggests the involvement of congenital CMV infection in a portion of children with ASD, although definite diagnosis was not obtained due to limited clinical data of the study subjects. (209 words)

Keywords: autism spectrum disorder, congenital cytomegalovirus infection, dried blood spots, dried umbilical cords

INTRODUCTION

Autism spectrum disorder (ASD) is a condition characterized by a clinical triad of impairment in social interactions, impairment in communications, and a restricted repertoire of activity and interests. In the Diagnostic and Statistical Manual of Mental Disorders-5 (DSM-5), a single diagnostic category, ASD encompasses previous diagnosis of autistic disorder, Asperger syndrome, childhood disintegrative disorder, and pervasive developmental disorders not otherwise specified, because they are not considered to be quite distinct disease entities but are a spectrum of syndrome with different severity of core symptoms and associated features [1].

The etiology of ASD is complex, and both genetic and environmental factors appear to be involved in its pathogenesis. An increase in the prevalence of ASD in recent years has fuelled concerns over possible environmental causes [2]. Intrauterine exposure to various pathogens has been associated with deficiency of fetal neurodevelopment, including ASD, in a number of previous studies [3].

ASD has been associated with children with congenital cytomegalovirus (CMV) infection who were symptomatic at birth [4-8]. Yamashita et al. reported that two of 7 children with symptomatic congenital CMV infection later developed ASD [9]. ASD has been also reported as a part of possible late manifestations in a child who was asymptomatic at birth. We have demonstrated congenital CMV infection in a child with ASD and mild

psychomotor retardation who developed epilepsy at 4 years of age by detecting CMV DNA in his umbilical-cord specimen [10]. To our knowledge, however, ASD has never been reported as the only late manifestation in children with asymptomatic congenital CMV infection, nor has any clinical study investigated the prevalence of congenital CMV infection in a single cohort of children with ASD. It is partly because diagnosis of congenital CMV infection usually requires specimens that were obtained within 3 weeks after birth: diagnosis of CMV infection at the time when they develop ASD never proves congenital infection.

Recently, retrospective diagnosis of congenital CMV infection became feasible by using dried blood-spot (DBS) specimens for mass screening of inborn metabolic errors [11] or preserved dried umbilical-cord specimens [12]. It has been Japanese tradition to keep dried umbilical cords as a symbol of mother-child bond for lifetime, which provided us with appropriate materials for retrospective diagnosis of congenital CMV infection in case DBS specimens have already been discarded [12].

In this study, we hypothesized that congenital CMV infection may play a pathological role in a proportion of children with ASD but no other obvious neurological manifestations, and demonstrated congenital CMV infection in two (7.4%) of 27 children with ASD in Nagasaki where the incidence of congenital CMV infection is 0.31%.

MATERIALS and METHODS

Study population

We recruited twenty-nine children with ASD who had been applied to psychoeducational interventions at facilities specified for developmental-behavioral disorders in Nagasaki, Japan. Diagnostic criteria was based on DSV-IV initially, and later reconfirmed by DSM-5. Diagnosis was made by a board-accredited pediatric neurologist (K.M.) and a board-accredited pediatrician (J.M.), both of whom have also received subspecialty training in developmental-behavioral pediatrics and have more than 10 years of practice experience in this field. Modified checklist for autism in toddlers (M-CHAT) and childhood autism rating scale (CARS) were also used to help diagnose ASD.

Two of the 29 recruited children were excluded because of significant perinatal events: one with severe asphyxia (#7) and the other with preterm delivery (#22). Ages of the remaining 27 children (24 males, 3 females) at enrollment ranged from 4 to 12 years (Table 1). Their birth years ranged from 1994 to 2003. Developmental quotient (DQ) scores were determined mainly by the Kyoto Scale of Psychological Development 2001 (KSPD) [13]; in some cases, Enjoji Scale of Infant Analytical Development (ESID)[14] or Kinder Infant Development Scale was recorded instead. Several patients were also tested with other methods, such as Wechsler Intelligence Scale for Children-III and the Tanaka-Binet Intelligence Scale. Auditory function was evaluated mostly with cognitive orientation response (COR). At recruitment in this study, written informed consent was obtained from their guardians. This study was approved by Institutional Review Board at Nagasaki

University.

Study design

DBS specimens were obtained from 20 children who were born in Nagasaki since December in 2000, and dried umbilical-cord specimens were from 7 children born before then.

For DNA extraction, six square pieces of 3 mm on one side were cut off from each DBS specimen. Similarly, a small piece (approximately 30 mg in weight) was cut off from each dried umbilical-cord specimen, using a clean cutter knife. In either case, blades were changed for each sample to avoid cross-contamination. DNA extraction and detection of CMV DNA by real-time polymerase chain reaction (PCR) were performed as described previously [12].

Newborn congenital CMV screening study

This study was conducted as a part of nationwide multicenter congenital CMV infection screening study [15]. A total of 3230 neonates who were born in six obstetric clinics in Nagasaki or referred to Nagasaki University Hospital from those clinics were enrolled from 2008 to 2010.

Urine samples were collected on a piece of FTA-Elute filter cards (Whatman). A 3-mm-diameter disc punched out from the urine filter was washed once, and used for real-time PCR, as described previously [15]. In case the urine specimen was positive for CMV DNA, another urine sample and a peripheral blood sample were obtained within 3 weeks after birth for

confirmation. DBS specimens were also prepared using the peripheral blood samples.

Statistical analyses

Statistical analyses were performed with Dr. SPSS II for Windows (IBM SPSS).

Fisher's exact test was used for the 2-by-2 table.

RESULTS

Clinical characteristics of study subjects

Among 27 subjects tested, 4 patients (#2, #3, #8, and #9) had mild-to-moderate hyperbilirubinemia that was treated with phototherapy, one patient (#1) had a fever, and another (#28) had a convulsion. No other patients had any perinatal symptom suggestive of congenital CMV infection. Three patients were born by Caesarean section for unknown reason (#10), breech presentation (#15), or previous Caesarean section (#19).

Three patients (#12, #26 and #28) had a history of convulsion, and one (#26) of them required anti-epileptic therapy. Diagnosis of epilepsy was not made in other two. Two patients (#18 and #21) were suspected of mild-to-moderate unilateral hearing impairment by COR but had not been confirmed by other auditory function tests in the respective ear (Table 1). Other subjects had no obvious neurological deficits.

Subjects with congenital CMV infection

CMV-DNA was detected in two (7.7%), #10 and #20. As described below,

neither of them was pointed out to have any abnormalities suggestive of congenital CMV infection at birth, and had any other neurological deficit except for ASD.

Patient #10 is a female born with birth weight of 2554 g at 38 weeks of gestation via Caesarean section for unknown reason in 2001. Otherwise, she had no significant family or perinatal history. She was afraid of strangers at 7 months of age, was able to walk by herself at 12 months of age, but was unable to speak a word until 24 months of age. Parents worried about her language delay since 18 months of age, but did not seek professional consultation until 34 months of age. At that time, a doctor (J.K.) specialized for developmental disorders demonstrated the following findings: Her eyes did not meet those of other persons. She could barely get communication with and hardly take turns with other persons. Although she could recognize surrounding situations and speak upon accepting the scene, her recognition of facial expression and emotion was poor. She liked reading both Japanese and English alphabets. She always acted at her own pace. She used gestures and pointing instead of verbal communication when she needed something. She also had self-injurious, restricted and repetitive behaviors, and showed unusual preoccupations with narrow interests. Her DQ, evaluated by KSPD, was 60 with Cognitive-Adaptive DQ 72 and Language-Social DQ 49. She had no history of convulsion or any other neurological problems including hearing impairment. Electroencephalogram (EEG) was normal.

Patient #20 is a male born with birth weight of 3424 g at 40 weeks of

gestation by vaginal delivery in 2001. He had no perinatal problems and his family history was unremarkable. His motor development had been up to date, but his language development had been relatively slow: he started to speak at 24 months of age and was able to speak two-word sentences at 36 months of age for the first time. Since 3 years of age, he easily panicked in unfamiliar settings or scenes, had difficulty in verbal and non-verbal communication, and exhibited signs of hyperesthesia. When he was referred to a doctor (K.M.) specialized for developmental disorders at 4 years of age, he was found to have failure of normal back-and-forth conversation, poorly integrated verbal and nonverbal communication, difficulties in making friends, echolalia, idiosyncratic phrases, and strong attachment to unusual objects and practices. His DQ, evaluated by ESID, was 84 with Motor-Body DQ 88, Motor-Hand DQ 104, Social-Basic life behavior DQ 96, Social-Interpersonal relations DQ 55, Language-Perception DQ 81 and Language-Production DQ 81 at that time. He had no history of convulsion or any other neurological problems including hearing impairment. Neither EEG nor brain imaging study was performed.

Upon environmental control, he became capable of attending public school and needed little therapeutic aid at 6 years of age. His intelligence quotient at that time was 94 (Tanaka-Binet).

Incidence of congenital CMV infection in Nagasaki

Among 3230 newborns enrolled in population-based congenital CMV infection screening program, 10 infants (0.31%) were found to be congenitally

infected with CMV. Their detailed clinicoepidemiological features will be described elsewhere (manuscript in preparation). While all of them excreted large amounts of CMV in their urine, viral loads in their peripheral blood were much lower (data not shown). When DBS specimens that were prepared at peripheral blood sampling were tested by real-time PCR, four (40%) of them were negative. Low sensitivity is also a problem when using dried umbilical-cord specimens (data not shown). Thus, urine-based screening should be more sensitive than retrospective diagnosis using DBS or dried umbilical-cord specimens.

In this study, we identified congenital CMV infection in two (7.4%) of 27 subjects with ASD. Such high incidence of congenital CMV infection in children with ASD is statistically significant ($p = 0.004$), even with such small number of subjects (Table 2).

DISCUSSION

Congenital CMV infection is the most important congenital infection in the developed countries. While 80-90% of congenitally CMV-infected infants are asymptomatic at birth, 10-20% of them later develop certain neurological problems, including sensorineural hearing loss, psychomotor retardation and epilepsy [16]. Autistic disorder is also considered a late-onset neuropsychiatric problem in children with congenital CMV infection [4-10].

Congenital infections have been associated with autistic disorder [3]. Among them, congenital rubella is a well-understood model for causing ASD [17-19]. Chess et al. reported that 7.4% of children with congenital rubella

developed autism [18]. They also demonstrated that while none of those who had no physical defect developed autism, 7.6% of those with one or two defect areas and 12.2% of those with three or four defect areas developed autism [18]. Similarly, autistic disorder has been associated with children with congenital CMV infection who were symptomatic at birth or had other major neurologic problem [4-10]. To the best of our knowledge, the two cases in this study were the first reported to develop ASD as the only neurodevelopmental manifestation in children with congenital CMV infection or any other congenital infections who were asymptomatic at birth, although it is impossible to confirm etiological relevance between ASD and congenial CMV infection.

In this study, we identified congenital CMV infection in two (7.4%) of 27 subjects with ASD. To demonstrate whether the incidence of congenital CMV infection in children with ASD is higher than that in general population, we took advantage of our recent population-based congenital CMV infection screening study (Table 2). Although there are two critical differences between the two studies, they will not affect our conclusion. First, specimens used for CMV detection were different: urine for the screening program and DBS or dried umbilical-cord specimens for this study. However, sensitivity should be much higher in urine-based screening than in our retrospective diagnosis as above [20]. We also confirmed higher sensitivity of urine-based screening in our prospective study: 40% of DBS specimens prepared from infants with congenital CMV infection were CMV DNA-negative in real-time PCR assays. Therefore, difference in specimens used may make bias against, but not in

favor of, our hypothesis. Second, birth years of subjects were different, 2008 to 2010 in the screening program and 1994 to 2003 in this study. In Japan, however, the incidence of congenital CMV infection (0.31%) is quite similar throughout the country during the period of 2008-2010 [15], and has been increasing since 1990's in Sapporo, Japan [15, 21]. Therefore, it is not unreasonable to assume 0.31% as the incidence of congenital CMV infection in Nagasaki Prefecture during the study period.

Other limitations in this study include a small sample size and limited clinical data of the study subjects. Despite the presence of only two cases of congenital CMV infection in the small study group consisting of 27 patients, the incidence (7.4%) of congenital CMV infection in the study group was significantly higher than that (0.31%) in the control group ($p = 0.004$). We recruited children with ASD who had no other major neurologic problem in this study. Those children were not admitted to a facility specialized for developmental disorders but attended designated clinics as outpatients. Only some of them underwent detailed neurological examinations such as electroencephalogram and imaging studies (cranial computed tomography scan or magnetic resonance imaging), because those tests were neither medically required nor too difficult to perform on them. Furthermore, their auditory function was evaluated mostly by COR but not by objective measures of auditory function such as auditory brainstem response; therefore, we might fail to identify patients with mild unilateral hearing loss.

If ASD is etiologically heterogeneous, including environmental factors like teratogens, it may not be unreasonable to assume that some

children develop ASD as a consequence of congenital CMV infection, possibly in combination with genetic susceptibility. Possible mechanisms whereby congenital CMV infection leads to ASD include direct teratogenic effects and indirect effects of inflammation on the developing brain [22, 23]. It is important to delineate how and to what extent congenital CMV infection is involved in the morbidity of autistic disorder, since pregnant women can reduce a risk of CMV infection by avoiding exposure to saliva and urine that might contain the virus. Larger-scale and more detailed studies are necessary to prove our hypothesis.

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Table 1. Profiles of study subject

Gender	Male	26
	Female	3
Age at recruitment (Years/Months)	3y9m - 12y7m	(mean 6y9m)
DQ	21 - 127	(mean 54)
	examined age (Y)	0y10m - 12y0m (mean 4y2m)
Intracranial imaging		
	Not analyzed	19
	Normal	6 (CT 3, MRI 3)
	Abnormal*1	4 (CT 0, MRI 4)
EEG	Not analyzed	20
	Normal	6
	Abnormal*2	3
Hearing impairment		
	Not pointed out	27
	Abnormal*3	2
CMV DNA	Negative	27
	Positive	2 (Cases 10, 20)

*1. The following findings are shown: (Methods)

case 7	pituitary hypoplasia	MRI
case 22	arachnoid cyst	MRI
case 23	arachnoid cyst	MRI
case 28	T2W1-high, T1W1-low spots in bilateral corona radiata	MRI

*2. The following findings are shown:

case 7	spikes (L>R)
case 11	frequent slow-wave components but no epileptiform activity
case 23	Irregularity in θ zone, sleep spindle waves in central frontal area

*3. The following findings are shown: (Methods)

case 18 unilateral hearing loss (mild - moderate) COR

case 21 unilateral hearing loss (mild - moderate) COR

Table 2. Comparison of CMV-DNA positivity between control and study groups

	CMV DNA		Total
	Neg	Pos	
Control group (Neonatal Screening)	3220	10 (0.31%)	3230
Study group (ASD patients)	25	2 (7.4%)	27

*Statistically significant ($p = 0.004$) difference was obtained in this 2-by-2 table by Fisher's exact test. There was no overlap between the two groups.