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Risk factors of early spontaneous abortions among Japanese: a matched case–control study

Sachiko Baba[1,2,3], Hiroyuki Noda[1,4], Masahiro Nakayama[3], Masako Waguri[5], Nobuaki Mitsuda[6], and Hiroyasu Iso[1,*]

[1]Public Health, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan [2]Center for International Relations, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suitai, Osaka 565-0871, Japan [3]Department of Clinical Laboratory Medicine and Anatomic Pathology, Osaka Medical Center and Research Institute for Maternal and Child Health, 840 Murodocho Izumi, Osaka 594-1101, Japan [4]Medical Center for Translational Research, Graduate School of Medicine, Osaka University, Suita-shi, Osaka 565-0871, Japan [5]Department of Maternal Medicine, Osaka Medical Center and Research Institute for Maternal and Child Health, 840 Murodocho Izumi, Osaka 594-1101, Japan [6]Department of Obstetrics, Osaka Medical Center and Research Institute for Maternal and Child Health, 840 Murodocho Izumi, Osaka 594-1101, Japan

*Correspondence address. Tel: +81-6-6879-3911; Fax: +81-6-6879-3919; E-mail: iso@pbhel.med.osaka-u.ac.jp

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BACKGROUND: No epidemiological studies have examined risk factors for early spontaneous abortions among Japanese women. In this matched case–control study, we investigated the associations of reproductive, physical, and lifestyle characteristics of women and their husbands with early spontaneous abortion <12 weeks of gestation.

METHODS: Information was collected through medical records for 430 cases of early spontaneous abortion and 860 controls of term delivery. Two controls were individual-matched to one case according to maternal age (±3 years) and calendar year of events (either early spontaneous abortion or delivery). Multivariable conditional odds ratios (ORs) and 95% confidence interval (CI) were calculated with conditional logistic-regression.

RESULTS: The risk of early spontaneous abortions was higher for women with a past history of early spontaneous abortions; OR was 1.98 (95% CI: 1.35, 2.89) for one previous spontaneous abortion, 2.36 (95% CI: 1.47, 3.79) for two, and 8.73 (95% CI: 5.22, 14.62) for three or more. Other factors also influence risk; an OR of 2.39 (95% CI: 1.26, 4.25) was found for women who smoked, and 1.65 (95%CI: 1.17, 2.35) for women working outside the home.

CONCLUSIONS: Our finding suggests that for Japanese women, smoking and working may be important public health issue targets for the prevention of early spontaneous abortions.

Key words: spontaneous abortion / Japanese / case–control study / employment / smoking

Introduction

Spontaneous abortions are serious life events. The frequency of early spontaneous abortions is estimated to be 10–15% of clinically recognized pregnancies and as many as 30% of clinically unrecognized pregnancies (Wilcox *et al.*, 1988).

Chromosomal abnormalities of the fetus and increasing maternal age are the major risk factors of early spontaneous abortions (Fretts *et al.*, 1995; Cunningham *et al.*, 2010). Ogasawara *et al.* reported that 50% of recurrent spontaneous abortions and 70% of sporadic spontaneous abortions among Japanese women were attributable to chromosomal abnormalities of the fetus. However, that study focused only on the relationship between embryonic karyotype and the number of previous spontaneous abortions without any adjustment for maternal age (Ogasawara *et al.*, 2000). Some other potential risk factors that have been considered for early spontaneous abortions include a previous history of early spontaneous abortions (Regan *et al.*, 1989), underweight (Helgstrand and Andersen, 2005; Maconochie *et al.*, 2007), obesity (Lashen *et al.*, 2004), uterine defects (Cunningham *et al.*, 2010) and lifestyle factors such as maternal smoking, alcohol consumption (Armstrong *et al.*, 1992; Larsen *et al.*, 2008) and employment status (Hemminki *et al.*, 1980). Yuan *et al.* reported that the frequency of spontaneous abortion among Japanese women tended to be higher among mothers who reported and smoking during pregnancy (spontaneous abortions for non-smokers constituted 17.9% for all pregnancies and 30.8% for smokers who smoke 1–10 cigarettes per day),

but they did not adjust for maternal age (Yuan *et al.*, 1994, in Japanese). Furthermore, Sado (1995, in Japanese) indicated that women in employment had higher risk of early spontaneous abortions, but did not adjust for maternal age, either. Few epidemiological investigations have been conducted concerning risk factors for early spontaneous abortions in Japan or other Asian countries.

Therefore, we conducted a case–control study matched for maternal age to evaluate the risk factors of early spontaneous abortions among Japanese women.

Methods

Study subjects

A hospital-based matched case–control study was conducted at Osaka Medical Center and Research Institute for Maternal and Child Health in Izumi city, Osaka, Japan which provides high-quality medical care using advanced technology, but also functions as the core hospital of the local area. The cases were selected consecutively. The case patients were all women who had identified early spontaneous abortions and had been hospitalized for a medical procedure with <12 weeks of gestation from January 2001 to December 2005 at the referral hospital. A total of 430 women were enrolled in this study. For the women who experienced two or more early spontaneous abortions in this period, the information for the last pregnancy was registered. Pregnancies were confirmed by positive human chorionic gonadotropin tests.

Of the 6169 women who underwent term deliveries from January 2001 to December 2005 in the referral hospital, 860 controls (2 controls per case) were randomly selected by matching the age of women (±3 years) and calendar year of events (either early spontaneous abortion or delivery).

The study was approved by the Ethics Committee of Osaka Medical Center and Research Institute for Maternal and Child Health and it was agreed that data collected through medical records would be used for this retrospective study, based on guidelines by the Council for International Organizations of Medical Science (1991).

Measurement of risk factors

The data were collected through medical records. We collected data on maternal age, height, pre-pregnancy weight, reproductive history, lifestyles and husbands' characteristics. Reproductive history included the number of past pregnancies, deliveries, induced abortions and early spontaneous abortions, as well as age at menarche, treatment for infertility (e.g. induction of ovulation, artificial insemination by husband, *in vitro* fertilization, other and unknown). Lifestyle habits included maternal smoking and amount of daily smoking, and drinking status (current drinker or not).

Maternal and husbands' smoking status was divided into non-smokers (never and ex-smokers) and current smokers of 1–19 cigarettes per day and ≥20 cigarettes per day. We categorized women who 'quit after learning of pregnancy' as 'current smoker' in maternal smoking status, because smoking before learning of a pregnancy could already have had an effect on early spontaneous abortions. We categorized homemaker as not employed, and the others as women employed because of the small numbers of women in each employment category (clerk, sales clerk, hairdresser, medical staff, teacher, children's nurse, service industry worker, student and others).

Statistical analysis

Data were analyzed using conditional logistic-regression model for a matched case–control study. Conditional odds ratios (ORs) with 95% confidence intervals (CIs) of early spontaneous abortions were calculated to examine the contribution of potential risk factors. Missing values were included as dummy variables in the analyses. To examine the effects a past history of spontaneous abortion could have all on potential risk factors, we also calculated the risk of early spontaneous abortions in terms of a combination of variables comprising past history of spontaneous abortion and other potential risk factors. We then checked for statistically significant interactions by using cross-product terms of past history of spontaneous abortion and other potential risk factors.

All analyses were two-tailed, and $P < 0.05$ was regarded as statistically significant. SAS, version 9.13 (SAS Institute, Inc., Cary, NC, USA) was used for all statistical analyses.

Results

Ages of cases were normally distributed from 17 to 45 years old. The mean (standard deviation: SD) age was 32.5 (5.20). There was no difference in the distribution of age by year (Table I).

Table II presents crude and multivariable conditional ORs and 95% CIs of early spontaneous abortions associated with reproductive, physical, lifestyle and husband characteristics. These results did not alter materially when a past history of early spontaneous abortion was excluded as an adjustment variable. There was a strong trend toward increased odds of an early spontaneous abortion among women with an increasing number of past early spontaneous abortions. The multivariable ORs (95% CI) of early spontaneous abortions were 1.98 (1.35, 2.89) for one, 2.36 (1.47, 3.79) for two and 8.73 (5.22, 14.62) for three or more previous spontaneous abortions.

Of the lifestyle characteristics, smoking and being employed were associated with the risk of early spontaneous abortions. Current maternal smoking of ≥20 cigarettes per day, i.e. heavy smoking for women, was associated with a two times higher risk of early spontaneous abortions; multivariable OR was 2.39 (1.26, 4.25). Women who were employed had an ~65% higher risk of early spontaneous abortions. Husband's age ≥40 years was associated with an ~60% higher risk of early spontaneous abortions in crude analysis, but in multivariable analysis, the association was no longer statistically significant. Past history of induced abortions, age at menarche, treatment for infertility, body mass index, alcohol consumption and husband's smoking were not associated with the risk of early spontaneous abortions.

We had a large number of missing data on husband's smoking and employment. When we restricted the analysis to women without missing data, the results did not alter substantially. For example, when the subjects without husband's smoking information were excluded from the analysis, the association between risk of early spontaneous abortion and employment remained statistically significant. The multivariable OR for being employed was 2.81 (95% CI: 1.40–5.65).

To examine the effect past history of early spontaneous abortions could have on the association between potential risk factors and risk of early spontaneous abortions, we further calculated the risks by combining the variable of past history with that of employment and with that of maternal smoking (Tables III and IV). The ORs of the combined variables were additive, and no interaction was observed between history of early spontaneous abortions and these associations (P for interaction was >0.20).

Table I Distributions of women with early spontaneous abortions (cases) and controls (term deliveries) by maternal age and calendar year.

	Maternal age	Calendar year					
		2001	2002	2003	2004	2005	Total (% of total)
Case ($n = 430$)	<25	6	7	2	8	2	25 (5.8)
	25–29	26	15	19	16	17	93 (21.6)
	30–34	27	37	24	36	28	152 (35.3)
	35–39	19	19	27	22	34	121 (28.1)
	≥40	4	9	5	12	9	39 (9.1)
	Total	82	87	77	94	90	430 (100.0)
Control ($n = 860$)	<25	12	14	8	17	5	56 (6.0)
	25–29	56	44	44	39	39	222 (25.8)
	30–34	58	69	46	72	67	312 (36.3)
	35–39	30	38	51	42	59	220 (25.6)
	≥40	8	9	5	18	10	50 (5.8)
	Total	164	174	154	188	180	860 (100.0)

Discussion

In this matched case–control study of Japanese women, we show that a past history of early spontaneous abortions, smoking and being employed are associated with increased risk of early spontaneous abortions. These relationships were not substantially altered in the multivariable adjustment models.

In this study, smoking ≥20 cigarettes per day was associated with a 2-fold or higher risk of early spontaneous abortions, which is in concordant with the finding of some previous studies (Armstrong *et al.*, 1992; Risch *et al.*, 1998; Maconochie *et al.*, 2007), but not all (Windham *et al.*, 1992; Rasch, 2003; Wisborg *et al.*, 2003). Smoking and a history of early spontaneous abortions had an additive effect on the risk of early spontaneous abortions. Maternal smoking has been found to lead to smoking-associated placental insufficiency and fetal hypoxia (Cnattingius and Nordström, 1996; Metwally *et al.*, 2008a,b). In Japan, the prevalence of smoking by young women has increased during the last half century. The prevalence of smoking by women aged 20–29 increased, from 6.6% in 1965 to 18.1% in 2008, and the corresponding findings for women aged 30–39 were 13.5 and 19.3% (Ministry of Health, 2006, in Japanese). Therefore, refraining from smoking by women of reproductive age may be an emerging public health issue in Japan.

In this study, we found that women in any type of employment had a 60% higher risk of early spontaneous abortions. We also found that being employed and history of early spontaneous abortions had an additive effect on the risk of early spontaneous abortions. Maternal employment, when it involves long hours or is physically demanding, may also affect other pregnancy outcomes such as preterm birth, small for gestational age, and maternal hypertension, but, in recent investigations, this has not been demonstrated for early spontaneous abortions (Mozurkewich *et al.*, 2000; Saurel-Cubizolles *et al.*, 2004). Several studies showed that women in specific occupations had higher risk for early spontaneous abortions. For example, one investigation of Finnish women in 1980 indicated that women in several occupational groups such as construction work and agriculture were at a higher risk than women without paid occupation, maybe from the physically demanding task (Hemminki *et al.*, 1980). Another investigation of Japanese pregnant women found that the proportion of early spontaneous abortions was significantly higher for women with any type of employment compared with women who were not employed: 13.1 versus 8.9% although they did not adjust for the age of women (Sado, 1995, in Japanese). However, to our knowledge, no studies from the other countries have shown any association between employment status and early spontaneous abortions (Savitz *et al.*, 1997; Maconochie *et al.*, 2007). A possible explanation for the magnitude of the effect that being employed has on the risk of spontaneous abortion for Japanese women may be the unequal division of house work between men and women. Time devoted to childcare by Japanese women is similar to that by European women (112 min in Japan versus 193 min in the UK and 118 min in Sweden), but that by Japanese men is much shorter than that by European men (corresponding figures are 25, 90 and 70 min) (OECD, 2001; Cabinet office, 2006). This suggests that Japanese women spend much more of their time and energy on a combination of housework and child-care than women in other regions. Further, menstrual disorders, an indicator of reproductive health, have been associated with work stress among full-time working women in China. Chinese women are reported to take primary responsibilities for their families even if they are in full-time employment, so that they too may have the double burden (Zhou *et al.*, 2010).

Neither being underweight nor overweight was strongly associated with the risk of early spontaneous abortions in our study. The proportions of lean and overweight women were similar to those reported by a national study (Ministry of Health, 2006, in Japanese), but the proportion of overweight women were far lower, and that of lean women were higher than those in other developed countries. The proportions of overweight women (BMI ≥ 25.0) were 19.9% in Japan, 56.6% in UK and 61.3% in the USA in most recent years, and those of underweight women (BMI < 18.5) were 10.4, 5.9 and 3.3% in these countries (World Health Organization). A Japanese population, as in our study, may thus be suitable for examining an effect of being underweight and the risk of spontaneous abortions observed in our study, but not suitable for examining an effect of being overweight.

Table II Crude and multivariable conditional ORs of spontaneous abortions associated with reproductive, physical, lifestyle and husband's characteristics.

Characteristics	Number		Crude OR (95% CI)	Multivariable OR (95% CI)
	Cases	Controls		
Reproductive variables				
Past history of spontaneous abortion				
None	232	632	1.0	1.0
1	77	124	1.75 (1.26–2.44)‡	1.98 (1.35–2.89)†
2	45	61	2.00 (1.32–3.03)‡	2.36 (1.47–3.79)‡
3 or more	76	42	5.36 (3.46–8.29)‡	8.73 (5.22–14.6)‡
Data missing	0	1	–	–
P for trend			<0.0001	
Past history of induced abortion				
None	360	748	1.0	1.0
1 or more	68	111	1.27 (0.91–1.76)	1.26 (0.84–1.89)
Data missing	0	1	–	–
Age at menarche (years)				
≤11	139	307	1.0	1.0
>12	149	272	1.26 (0.95–1.66)	1.23 (0.89–1.72)
Data missing	142	281	–	–
Treatment for Infertility (Assisted Reproductive Technology)				
No	372	748	1.0	1.0
Yes	55	103	1.08 (0.75–1.54)	1.00 (0.67–1.49)
Data missing	3	9	–	–
Physical variables				
Body mass index (kg/m^2)				
<18.5	73	152	0.99 (0.72–1.36)	0.86 (0.59–1.25)
18.5–24.9	310	637	1.0	1.0
≥25.0	47	71	1.34 (0.91–1.96)	1.40 (0.87–2.25)
P for trend			0.31	0.23
Lifestyle variables				
Maternal smoking status				
Non-smoker	339	722	1.0	1.0
Current smoker (1–19/day)	54	101	1.24 (0.83–1.85)	1.30 (0.84–2.02)
Current smoker (≥20/day)	32	36	1.99 (1.18–3.35)†	2.39 (1.26–4.53)*
Data missing	5	1	–	–
P for trend			0.03	0.02
Current drinker				
No	346	759	1.0	1.0
Yes	75	120	1.32 (0.97–1.82)	1.04 (0.71–1.53)
Data missing	9	1	–	–
Employment				
No	174	357	1.0	1.0
Yes	146	165	1.64 (1.21–2.23)†	1.65 (1.17–2.35)†
Data missing	111	338	–	–
Husband's variables				
Husband age (year)				
<29	98	225	1.0	1.0
30–39	246	505	1.29 (0.91–1.87)	1.14 (0.75–1.74)
≥40	86	130	1.98 (1.24–3.23)†	1.65 (0.94–2.88)
P for trend			0.015	0.017

Continued

Table II *Continued*

Characteristics	Number		Crude OR (95% CI)	Multivariable OR (95% CI)
	Cases	Controls		
Husband's smoking status				
Non-smoker	114	205	1.0	1.0
Current smoker (1–19/day)	65	97	1.24 (0.83–1.85)	1.23 (0.78–1.96)
Current smoker (≥20/day)	72	103	1.30 (0.88–1.92)	1.30 (0.82–2.06)
Data missing	179	455	–	–
P for trend			0.35	0.47

95% CI, 95% confidence interval; OR, conditional odds ratio; –, valid OR was not calculated. Matched for calendar year of the event and mother age (±3 years). Multivariable conditional OR was adjusted for past spontaneous abortion history, past induced abortion history, treatment for infertility, body mass index, smoking status, drinking status, employment, husband's age and husband's smoking.
*$P < 0.05$.
†$P < 0.01$.
‡$P < 0.001$.

The lack of association between being underweight and the risk of early spontaneous abortions observed in our study was consistent with the findings from two previous studies (Stein and Kline, 1991; Helgstrand and Andersen, 2005), but one case–control study reported a significant association between underweight and spontaneous abortions; the multivariable OR was 1.72 and 95% CI was 1.17, 2.53; Maconochie *et al.*, 2007).

The influence of obesity on early spontaneous abortions has been widely investigated. Obesity may increase the risk of spontaneous abortions by an adverse influence on the embryo, the endometrium or both via leptin (Metwally *et al.*, 2007, 2008a,b), but there are few studies on conception without any assisted reproductive technology. Metwally *et al.* (2008a,b) show in their meta-analysis that obesity may increase the risk of spontaneous abortions, but they included middle-term abortions until 19 weeks of gestation. On the other hand, Maconochie *et al.* (2007) showed that obesity did not increase the risk of spontaneous abortions <13 weeks in a large population-based case–control study.

This study has a number of potential limitations. First, the study subjects were sampled in a single hospital. In Japan, patients can choose the hospital in which they are treated according to their own preferences, while medical costs are uniform under the national medical insurance system. Women seeking advanced medical treatment, as well as pregnant women at normal risk or without complications, are more likely to visit our hospital. Therefore, the subjects in our study may have been biased toward high-risk pregnant women. Secondly, the case patients were defined as the women who were hospitalized for a medical procedure. A woman who had a complete spontaneous abortion without any additional treatment would not be included. We may evaluate the risk of missed abortions, but not all types of early spontaneous abortions. Thirdly, the data were collected retrospectively through medical records. Therefore, we did not measure caffeine, socio-economic status or other potential confounding variables (Cnattingius *et al.*, 2000; Luo *et al.*, 2006). However, none of these variables are established risk factors for early spontaneous abortions, so that the

Table III Crude and multivariable conditional ORs of early spontaneous abortions associated with combination of previous early spontaneous abortion and employment.

	Number		Crude OR (95% CI)	Multivariable OR (95% CI)
	Cases	Controls		
Previous abortions (−), Employment (−)	97	360	1.0	1.0
Previous abortions (−), Employment (+)	88	137	1.56 (1.08–2.26)*	1.50 (1.01 –2.24)*
Previous abortions (+), Employment (−)	77	97	2.28 (1.54 –3.39)‡	2.40 (1.56 –3.7)‡
Previous abortions (+), Employment (+)	58	29	5.16 (2.98 –8.94)‡	5.56 (3.04 –10.1)‡
Data missing	110	337	–	–

95% CI, 95% confidence interval; OR, conditional odds ratio; –, valid conditional OR was not calculated. Previous abortion (+) includes 1, 2 and 3 or more previous abortions. Matched for calendar year of the event and mother age (±3 years). Multivariable conditional OR was adjusted for past induced abortion history, treatment for infertility, body mass index, smoking status, drinking status, husband's age and husband's smoking.
*$P < 0.05$.
†$P < 0.01$.
‡$P < 0.001$.

Table IV Crude and multivariable conditional ORs of early spontaneous abortions associated with combination of previous early spontaneous abortion and maternal smoking.

	Number		Crude OR (95% CI)	Multivariable OR (95% CI)
	Cases	Controls		
Previous abortion (−), Maternal smoking (−)	181	525	1.0	1.0
Previous abortion (−), Maternal smoking (+)	50	107	1.42 (0.97 −2.09)	1.39 (0.89 −2.17)
Previous abortion (+), Maternal smoking (−)	158	197	2.43 (1.83 −3.22)‡	2.95 (2.14−4.05)‡
Previous abortion (+), Maternal smoking (+)	36	30	3.53 (2.10 −5.95)‡	4.31 (2.37 −7.86)‡
Data missing	5	1	−	−

95% CI, 95% confidence interval; OR, conditional odds ratio; −, valid conditional OR was not calculated. Previous abortion (+) includes 1, 2, and 3 or more previous abortions. Maternal smoking (+) includes smoking (1 − 19/day) and (≥20 /day). Matched for calendar year of the event and mother age (± 3 years). Multivariable conditional OR was adjusted for past induced abortion history, treatment for infertility, body mass index, smoking status, drinking status, husband's age, and husband's smoking.
*$P < 0.05$.
†$P < 0.01$.
‡$P < 0.001$.

residual confounding may not be large. Fourthly, there were a large number of missing data for employment and husband's smoking, but the results did not change substantially among the subjects without these missing data.

In conclusion, our study suggested that smoking and being employed, as well as a past history of early spontaneous abortions, were associated with early spontaneous abortions among Japanese women. Our findings suggest that smoking and/or working women may be important public health targets for the prevention of early spontaneous abortions in Japan.

Authors' roles

S.B. analyzed and interpreted the data, drafted the manuscript and provided statistical expertise. H.N. and M.N. designed the study's analytic strategy. M.W. and N.M. critically revised the manuscript. H.I. conceived and designed the study, acquired and interpreted the data and critically revised the manuscript.

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Original Article

Immune response to *Haemophilus influenzae* type b conjugate vaccine in preterm infants

Kennosuke Tsuda,[1] Shiho Iwasaki,[2] Haruko Horiguchi,[1] Masaaki Mori,[3] Shigeru Nishimaki,[2] Kazuo Seki,[1] Masataka Taguri,[4] Shumpei Yokota[2] and Naruhiko Ishiwada[5]

[1]*Perinatal Center for Maternity and Neonate,* [2]*Department of Pediatrics, Yokohama City University Medical Center, Department of* [3]*Pediatrics,* [4]*Biostatistics and Epidemiology, Yokohama City University Graduate School of Medicine, Yokohama, Kanagawa and* [5]*Department of Pediatrics, Chiba University Hospital, Chiba, Japan*

Abstract ***Background***: *Haemophilus influenzae* type b (Hib) vaccine became available for use in Japan in December 2008. The aim of the present study was to evaluate the immunogenicity of Hib vaccine in Japanese preterm infants.

Methods: Serum samples were obtained from 54 preterm infants before the first vaccination and 1 month after the third. Anti-polyribosylribitol phosphate (PRP) antibodies were measured using an enzyme-linked immunosorbent assay method. Antibody positivity was defined as levels >1 μg/mL.

Results: Of the 54 preterm infants, 46 (85.2%) achieved antibody levels >1 μg/mL. This compares with the 92.4% reported in full-term infants. The antibody seroconversion rate of infants starting vaccination at 2 months of age was close to being significantly lower than when vaccination was started at 3 months of age ($P = 0.060$). In addition, the percentage of infants achieving a positive response in the group with a history of antenatal steroid exposure was significantly higher than in those not exposed ($P = 0.046$). Thus, risk factors for lower Hib antibody concentrations after three doses of vaccine were age at first vaccination and lack of use of antenatal steroids.

Conclusions: There is a possibility that perinatal factors and the environment unique to preterm infants are related to their lower antibody positivity rates compared to full-term infants. It may therefore be preferable to modify the proposed immunization schedule.

Key words anti-polyribosylribitol phosphate antibody, *Haemophilus influenzae* type b, immunogenicity, preterm infant, vaccine.

Haemophilus influenzae is one of the leading causes of pediatric bacterial meningitis in Japan. *Haemophilus influenzae* consists of both unencapsulated and encapsulated strains. Of the latter, *Haemophilus influenzae* type b (Hib), which possesses polyribosylribitol phosphate (PRP), is a major cause of invasive infections, such as meningitis. After its first introduction in 1988, Hib vaccine has been widely used in the USA since 1990. As a consequence, the prevalence of infectious diseases caused by Hib has decreased dramatically.[1–5] In December 2008, Hib vaccine was introduced into Japan.

Correspondence: Kennosuke Tsuda, Perinatal Center for Maternity and Neonate, Yokohama City University Medical Center, 4-57 Urafune-cho, Minami-ku, Yokohama, Kanagawa 232-0024, Japan. Email: tsudaken@bk2.so-net.ne.jp

Departments and institutions in which the work was performed:

Department of Pediatrics, Yokohama City University School of Medicine

Perinatal Center for Maternity and Neonate, Yokohama City University Medical Center

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For Hib vaccine use in Japan, clinical trials were carried out in 19 facilities, on 200 babies over the period February 2000–February 2001. Good antibody responses were obtained.[6] The subjects investigated in these clinical trials were all full-term infants. However, there are thus far no data on the immunogenicity of the Hib vaccine in preterm infants in Japan, and similarly, there appear to be few reports in other countries. The aim of the present study was therefore to evaluate vaccine immunogenicity in preterm infants by measuring antibody titers before and after vaccination.

Methods

Subjects

We conducted a prospective cohort study. Parents of premature infants (<37 weeks gestation; inpatients of the neonatal intensive care unit of Yokohama City University Hospital and Yokohama City University Medical Center) were approached between December 2008 and October 2009. A total of 54 infants were enrolled in the study after informed parental consent was

obtained. The study protocol was approved by the Yokohama City University Ethics Committee.

Vaccine

In this study, we sought to follow a cohort of preterm infants vaccinated with PRP-T (ActHIB, Sanofi Pasteur, France). In accordance with the manufacturer's recommendations, Hib vaccine was injected subcutaneously in a total volume 0.5 mL in the left or right arm. We started vaccination when the infants were aged 2–6 months, with three injections at intervals of 4 weeks. First immunization was delayed if an infant was still mechanically ventilated or clinically unstable or if there were concerns about intercurrent sepsis.

Immunogenicity studies

Blood was obtained by venepuncture prior to the first dose of Hib vaccine and 1 month after the third dose. The blood was centrifuged on return to the laboratory and serum stored at −20°C until serological tests were performed. Anti-PRP antibody titers were determined using Bindazyme Anti-Haemophilus B Enzyme Immunoassay Kits (Binding Site, Birmingham, UK). This method was the same as that described by Ocaktan *et al.*[7]

Statistical analyses

Statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL, USA). Gestational age and birthweights are reported as medians (range). Anti-PRP antibodies were converted by logarithmic transformation and are reported as geometric mean titers (GMT) with 95% confidence intervals. Concentrations <0.15 μg/mL were allocated a value of 0.08 for purposes of calculation. Antibody positivity was defined as ≧1 μg/mL, and negativity as <1 μg/mL. This is based on the report that PRP antibody titers of patients who developed Hib systemic infection were never ≧1 μg/mL (by enzyme-linked immunosorbent assay) in the acute phase.[8]

Analyses of the influence of different perinatal factors on vaccine immunogenicity in premature infants were performed. The *t*-test was used for comparing continuous variables (gestational age and birthweights) and the χ^2-test (Yates's corrected) or Fisher's exact test for comparing categorical variables in antibody-positive and -negative patients. The following factors were included in the analyses: age at first vaccination, gestational age, birthweight, maternal infection, neonatal infection, antenatal steroids, postnatal steroids, and breast-feeding (exclusive/non-exclusive).

We defined maternal infection on the basis of diagnoses recorded by clinical caregivers. Indicators of maternal infection were a clinical diagnosis of chorioamnionitis, maternal temperature during labor greater than 38°C, foul-smelling amniotic fluid during the admission for delivery, or maternal sepsis. Definition of neonatal infection was suspected cases of infection from blood tests or some symptoms. Blood culture was not necessarily positive. Differences were considered significant at $P < 0.05$.

Results

All 54 preterm infants completed the study. Vaccination was started in 23 infants (42.6%) at 2 months, 18 (33.3%) at 3 months, seven (13.0%) at 4 months, two (3.7%) at 5 months and the remaining four (7.4%) at 6 months of age.

Table 1 Influence of perinatal factors on rate of antibody seroconversion

	Antibody		*P*
	Negative	Positive	
	n = 8	*n* = 46	
	n (%)		
Sex (male)	5 (62.5)	31 (67.4)	1.000
Small for gestational age	3 (37.5)	19 (41.3)	1.000
Age at first vaccination (2 months)	6 (75.0)	17 (37.0)	0.060
Maternal infection (+)	0 (0.0)	7 (15.2)	0.577
Neonatal infection (+)	2 (25.0)	15 (32.6)	1.000
Antenatal steroids (+)	2 (25.0)	37 (80.4)	0.046*
Postnatal steroids (+)	1 (12.5)	1 (2.2)	0.277
Exclusive breast-feeding	3 (37.5)	22 (47.8)	0.711
Gestational age† (weeks of gestation)	31.0 (4.2)	29.4 (3.1)	0.197
Birthweight† (g)	1419 (634)	1173 (380)	0.148

*$P < 0.05$ was considered as statistically significant. †Mean (SD).

The median gestational age was 30 weeks (range 23–36). The median birthweight was 1.11 kg (range 0.46–2.34) with 66.7% boys. The proportion of small-for-gestational-age (SGA) infants was 22 cases (40.7%). Infection was documented in 13.0% of mothers and 31.5% of infants. Steroids were given antenatally to 72.2% of mothers and two (3.7%) of the infants, in the latter for 10 days and 32 days, respectively, for the treatment of hypoglycemia. Nearly half of the infants (46.3%) were exclusively breast-fed. There were no antibody-positive cases before vaccination, but after three doses of vaccine, GMT had increased from 0.06. to 2.89 μg/mL ($P < 0.001$). The seroconversion rate was 85.2% (46 of the 54 cases). The responders (positive group) were compared with the non-responders (negative group) regarding perinatal factors which might have affected the outcome (Table 1). Dividing infants into two groups according to age at first vaccination of 2 months vs >3 months revealed that the seroconversion rate of the former was close to being significantly worse than the latter ($P = 0.060$). In addition, the rate of seroconversion after vaccination of infants with a history of antenatal exposure to steroids was higher than those without ($P = 0.046$). No statistical significance was observed between antenatal steroids and bronchopulmonary dysplasia. And there was also no trend between bronchopulmonary dysplasia and seroconversion rate (data not shown). No other perinatal factors were found to significantly influence the seroconversion rate. Side-effects after vaccination were rare, with only one case of local redness. There was no case of Hib disease among the infants.

Discussion

Vaccines against Hib became available in Japan only quite recently. Thus, there are only sparse data available on the immunogenicity of these Hib conjugate vaccines. To the best of our knowledge, the present study is the first to examine the immunogenicity of Hib vaccines in Japanese preterm infants. Studies

comparing immunogenicity in preterm and full-term infants have been described in the literature in other countries, although these varied in vaccine type and vaccination schedule.[9–11] Those studies documented that preterm infants responded less frequently than full-term babies and with lower anti-PRP antibody concentrations following Hib vaccination, such that fewer achieved antibody titers above the conventionally accepted protective concentrations.[12] Preterm Japanese infants were found to have significantly lower GMT of anti-PRP antibody even after three doses of vaccines compared to full-term infants:[6] these values were 2.89 compared with 9.68 μg/mL, with lower seroconversion rate of 85.2% vs 92.4%. Our immunogenicity data reported here are generally consistent with those of other published studies. In the present study, we aimed to evaluate the immunogenicity of vaccinating preterm infants against Hib, focusing on those who had required perinatal treatment. Risk factors for lower Hib antibody titers after three doses of vaccine were identified as age at first vaccination and lack of use of antenatal steroids. Above all, we particularly focused on age at first vaccination, where there appear to have been no studies thus far. We found that seroconversion by infants first vaccinated at 2 months of age was close to being significantly less frequent than when the first vaccination was at 3 months of age. In Japan, infants <7 months of age are offered the first Hib vaccination of the series at 2 or 3 months of age. According to the recommendations of the American Academy of Pediatrics Committee on Infectious Diseases, medically stable preterm infants should receive full doses of acellular pertussis, diphtheria, tetanus, Hib, hepatitis B, poliovirus, and pneumococcal conjugate vaccines at a chronological age consistent with the schedule recommended for full-term infants.[13] Vaccine dosages normally given to full-term infants should not be reduced or divided into separate doses when given to preterm infants and those with low birthweight. Moreover, it was shown that all vaccines routinely recommended during infancy are safe for use in preterm infants and, although the immunogenicity of some childhood vaccines may be decreased in the smallest preterm infants, the antibody concentrations achieved are usually protective.[14–16]

In addition to the lesser ability of B cells to produce antibodies in preterm infants[17] and the immaturity of the neonatal immune system, perinatal factors are also considered to contribute to the lower antibody production in preterm compared to full-term infants. Previous studies have shown that the presence of chorioamnionitis or intrauterine infection influences the fetal and neonatal immune system via a mechanism mediated by cytokines.[18] Antenatal steroid restrains T cell proliferation.[19] However, the state of knowledge on immune status of newborns is still poor, especially for preterm infants. It is also not yet clear whether perinatal medication affects the neonatal immune system.

As a matter of course, preterm infants, particularly those that have to be admitted to neonatal intensive care units, are less likely to be exposed to bacteria cross-reacting with Hib.[20] Consequently, it is difficult to induce natural booster effects and their anti-PRP antibody concentrations remain low.

The reason for the tendential correlation between age at first vaccination and vaccine responses in this study is not yet established. Similarly, the reason for significant association between antenatal steroid use and antibody responses is also unclear. One study demonstrated previously that the seroconversion rate did not differ significantly whether or not infants were exposed to antenatal steroids.[11] The number of samples in the present study was limited. The aim of this study was to report that Japanese preterm infants have lower seroconversion rate. Further studies are needed to confirm these findings.

The low antibody concentration itself can be a risk factor for Hib infection. In general, it is recommended to start vaccination for Hib from 2 months of age because relatively high levels of transplacentally acquired anti-PRP antibodies fall over the first months of life to very low levels by around 6 months of age.[21,22] Moreover, preterm infants are less likely to have maternal antibody – particularly very premature (≤32 weeks) infants. Preterm infants are less exposed to horizontal transmission. Hence, it might be better to start vaccination at 3 months of age. When starting at 2 months of age, it is recommended that the number of vaccinations be increased, or the interval between vaccinations extended.[23] However, it should be noted that preterm infants living with siblings should still start vaccination earlier because they are more likely to be exposed to other carriers, especially in group nursing.

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Brief Communication

Clinical and molecular aspects of Japanese children with medium chain acyl-CoA dehydrogenase deficiency

Jamiyan Purevsuren [a,*], Yuki Hasegawa [a], Seiji Fukuda [a], Hironori Kobayashi [a], Yuichi Mushimoto [a], Kenji Yamada [a], Tomoo Takahashi [a], Toshiyuki Fukao [b,c], Seiji Yamaguchi [a]

[a] Department of Pediatrics, Shimane University Faculty of Medicine, Izumo 693-8501, Japan
[b] Department of Pediatrics, Graduate School of Medicine, Gifu University, Gifu, Gifu 501-1194, Japan
[c] Medical Information Sciences Division, United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, Gifu, Gifu 501-1194, Japan

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ABSTRACT

We report the outcome of 16 Japanese patients with medium chain acyl-CoA dehydrogenase deficiency. Of them, 7 patients were diagnosed after metabolic crisis, while 9 were detected in the asymptomatic condition. Of the 7 symptomatic cases, 1 died suddenly, and 4 cases had delayed development. All 9 patients identified by neonatal or sibling screening remained healthy. Of 14 mutations identified, 10 were unique for Japanese, and 4 were previously reported in other nationalities. Presymptomatic detection including neonatal screening obviously improves quality of life of Japanese patients, probably regardless of the genotypes.

1. Introduction

Medium chain acyl-CoA dehydrogenase deficiency (MCADD) (MIM #201450) is an autosomal recessive inherited metabolic disorder of mitochondrial fatty acid oxidation. The number of MCADD patients has recently become larger in Japan with the spread of acylcarnitine analysis using tandem mass spectrometry (MS/MS). The disease frequency was estimated to be approximately 1:100,000 in Japan according to a newborn screening pilot study of 1.57 millions babies (unpublished report). Clinical symptoms of MCADD are heterogeneous, ranging from asymptomatic to severe handicaps followed by metabolic crisis or sudden unexpected death (SUD) [1,2]. Approximately 20% of previously undiagnosed patients die during their first metabolic decompensation [3–7]. Blood acylcarnitine, urinary organic acid analyses, MCAD activity and mutation analyses are major tools for diagnosis of MCADD. A common c.985A>G mutation has been reported in 80–90% of Caucasian patients [8–16] while c.449–452delCTGA mutation was identified in 45% of mutant alleles in Japanese patients with MCADD [17]. In recent years, the detection incidence of the presymptomatic patients with MCADD has increased since the neonatal mass screening was expanded in Japan. However, there are few reports of the outcomes of the Japanese patients. Herein, we report the relation of clinical onsets, genotypes and outcomes of 16 Japanese children with MCADD, and 4 heterozygote carriers, which were analyzed in Shimane University.

2. Subjects and methods

2.1. Subjects

Sixteen Japanese patients with MCADD from 15 unrelated families, including previously reported 9 cases [17], and 4 carriers were studied (Table 1). The patients were analyzed for confirmation of diagnosis in Shimane University from 2001 to 2011. Of them, 8 (cases 8 to 16) were identified by neonatal mass screening, 7 (cases 1 to 7) were diagnosed after metabolic crisis, and 1 was detected by sibling screening. Cases 2 and 8 were siblings, and cases 19 and 20 were parents of case 16. Diagnosis of the patients was confirmed by urinary organic acid, blood acylcarnitine and mutation analyses.

2.2. Mass spectrometric analysis

Acylcarnitines in blood spots on filter paper were analyzed by a method standardized for neonatal mass screening using MS/MS, an API 3000 instrument (Applied Biosystems, Foster City, CA, USA) [8,18]. Urinary organic acids were analyzed using the solvent extraction method by the QP 2010 capillary GC/MS system (Shimadzu Co., Ltd., Kyoto, Japan) [19]. The determination of test values was assessed using reference values set at the Shimane University.

* Corresponding author at: Department of Pediatrics, Shimane University Faculty of Medicine, 89-1 Enya, Izumo, Shimane 693-8501, Japan. Fax: +81 853 20 2215.
E-mail address: jamiyan@med.shimane-u.ac.jp (J. Purevsuren).

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Table 1
Clinical and genetic characteristics of Japanese patients with MCAD deficiency.

Patient	Sex	Age at onset	Age at diagnosis	Neonatal screening	Primary clinical symptoms	Hypoglycemia	Hyperammonemia	Tandem MS		GC/MS (RPA%)		Genotype		Outcome
								C8 <0.35μM	C8/C10 (<3)	HG	SG	Allele 1	Allele 2	
Symptomatic group														
1	F	1y	1y	–	Cardiopulmonary arrest, dyspnea, poor feeding	(+)	(−)	4.52	8.69	n.a	n.a	IVS4+1G≥A	c.422A≥T (Q116L)	Sudden death
2 [a] *	M	1y 4m	1y 4m	–	Gastroenteritis, seizures	(+)	(−)	3.33	17.53	9.9	15.3	c.449–452delCTGA	c.449–452delCTGA	Severe handicapped
3 [a]	M	8m	8m	–	Cardiopulmonary arrest	(n.a)	(+)	5.97	3.49	11.1	44.5	c.449–452delCTGA	c.157C>T (R28C)	Developmental delay
4	F	1y 1m	1y 1m	–	Developmental regression	(+)	(+)	7.00	21.00	14.7	112.2	del. ex 11–12	del. ex 11–12	Developmental delay
5 [a]	F	2y 2m	2y 2m	–	Cold, gastroenteritis	(+)	(−)	1.71	15.55	n.a	n.a	c.449–452delCTGA	c.449–452delCTGA	Developmental delay
6 [a]	F	1y 3m	1y 3m	–	Unconsciousness, apnea, vomiting	(n.a)	(−)	n.a	n.a	n.a	n.a	del. ex 11–12	del. ex 11–12	Normal
7 [a]	F	1y 7m	1y 7m	–	Unconsciousness, fever	(+)	(+)	4.12	10.05	6.1	6.4	c.275C>T (P67L)	c.157C>T (R28C)	Normal
Asymptomatic group														
8 [a] *	M	–	5y 5m	–	Normal	(−)	(−)	1.37	39.14	n.a	n.a	c.449–452delCTGA	c.449–452delCTGA	Normal
9 [a]	F	–	5d	+	Normal	(−)	(−)	5.92	11.38	12.9	14.8	c.1085G>A (G337E)	c.843A>T (R256S)	Normal
10	F	–	5d	+	Normal	(−)	(−)	5.37	12.49	6.33	39.88	c.449–452delCTGA	c.157C>A (R28H)	Normal
11	M	–	5d	+	Normal	(−)	(−)	4.82	13.03	15.3	3.8	IVS3+2T≥C	c.843A>T (R256S)	Normal
12	F	–	5d	+	Normal	(−)	(−)	4.04	14.96	n.a	n.a	c.449–452delCTGA	c.212G≥A (G46D)	Normal
13 [a]	F	–	5d	+	Normal	(−)	(−)	2.78	15.44	11.5	5.9	c.449–452delCTGA	c.134A>G (Q20R)	Normal
14	F	–	5d	+	Normal	(−)	(−)	2.59	10.00	3.08	3.20	c.1085G≥A (G337E)	c.1184A≥G (K370R)	Normal
15	M	–	5d	+	Normal	(−)	(−)	2.58	8.32	(−)	1.50	c.449–452delCTGA	IVS3+5G≥A	Normal
16 [a]	M	–	5d	+	Normal	(−)	(−)	0.49	3.77	9.7	(−)	c.449–452delCTGA	c.820A>C (M249V)	Normal
Carrier group														
17	M	–	5d	+	Normal	(−)	(−)	0.44	1.02	(−)	(−)	c.845C>T (P257L)	n.d	Normal
18	F	–	4m	–	Eczema	(−)	(−)	0.51	0.88	(−)	(−)	c.843A>T (R256S)	n.d	Normal
19	M	–		–	Normal	(−)	(−)	0.37	1.00	n.a	n.a	c.449–452delCTGA	n.d	Normal
20	F	–		–	Normal	(−)	(−)	0.20	0.95	n.a	n.a	c.820A>C (M249V)	n.d	Normal

[a]: Purevsuren et al. [17] reported; *: siblings; sex: M, male; F, female; age: y, year; m, month; d, day; +, involved to neonatal mass screening; (−), not detected; n.a, not available; RPA%, relative peak area percentage; HG, hexanoylglycine; SG, suberylglycine; novel mutations are underlined.

2.3. DNA sequencing of gene, acyl-CoA dehydrogenase, medium chain (ACADM)

Genomic DNA was purified from the patients' fibroblasts or blood filter papers using the QIAamp DNA Micro Kit (Qiagen GmbH, Hilden, Germany). Mutation analysis on genomic DNA was performed by PCR for each exon and its intron boundaries followed by direct sequencing [17].

Informed consent to perform DNA analysis was obtained from the parents of the patients. This study was approved by the Ethical Committee of the Shimane University Faculty of Medicine.

3. Results

3.1. Clinical features of patients

The clinical features of 16 Japanese patients with MCADD and 4 carriers (9 males and 11 females) are summarized in Table 1, including previously reported cases [17]. All 7 patients that were diagnosed after metabolic crisis were born before the initiation of newborn screening in their local area. The mean age at onset of the symptomatic cases was 1 y 3 m (range: 8 m to 2 y 2 m). The symptomatic patients were all in good general health with normal development until metabolic crisis. Metabolic crises were triggered by common cold or gastroenteritis in 5 cases. One of them died of SUD. Four cases had mild to severe handicaps, and 2 cases developed normally. The patients who were identified by neonatal screening remain healthy at this time.

3.2. Biochemical results of patients

The results of mass spectrometric analysis are shown in Table 1. Blood acylcarnitine analysis was available in 15 of the 16 patients. Octanoylcarnitine (C8) and octanoyl:decanoylcarnitine (C8/C10) ratio were assessed for detection of MCADD. Marked elevation of C8 and C8/C10 was observed in 14 cases (1.37–7 μmol/L), and slight elevation of C8 and C8/C10 (0.49 μmol/L and 3.77) was found in one case (case 16). The level of C8 was also mildly elevated in 3 (0.44, 0.51 and 0.37 μmol/L, respectively) of the 4 carriers while C8/C10 value was under cut-off (1.02, 0.88 and 1.00). Case 20, who is a mother of case 16, showed no abnormal findings.

Urinary organic acids were analyzed in 11 cases with MCADD and 4 carriers. Both hexanoylglycine and suberylglycine were elevated in 9 patients, and hexanoylglycine or suberylglycine was increased in one case each. However, neither hexanoylglycine nor suberylglycine was identified in the carriers.

3.3. Mutations in acyl-CoA dehydrogenase, medium chain (ACADM) gene

Fourteen types of mutations were identified in 30 independent alleles, 7 of which were novel. These included three types of splice site alterations (IVS3+2T>C, IVS3+5G>A and IVS4+1G>A), and four missense mutations (G46D, Q116L, G337E and K395R). These novel mutations were not detected in 120 alleles from unaffected Japanese individuals. All mutations are summarized in Table 1, together with previously reported cases (cases 2, 3, 5–9, 13 and 16) [17]. A c.449–452delCTGA [20,21] was detected in 10 (33.3%) of 30 independent alleles (2 cases with homozygous and 6 cases with compound heterozygous). A homozygous large deletion including exons 11 and 12 [22] was identified in 4 (13.3%) alleles. R28C (2/30 alleles), R256S (2/30 alleles), P67L (1/30 alleles), M249V (1/30 alleles) and G337E (1/30 alleles) were also observed (Table 1) [9,17,22].

4. Discussion

We investigated the relationship between clinical and molecular spectrums of 16 Japanese patients with MCADD. While symptomatic patients remained undiagnosed until metabolic crisis, asymptomatic patients were identified by neonatal mass screening (8 cases), or by sibling screening (1 case). Most of the symptomatic cases developed metabolic crisis associated with hypoglycemia triggered by common infection and prolonged fasting [3,4]. Those patients had poor outcomes such as mild to severe impairments or SUD. However, expansion of blood acylcarnitine analysis using MS/MS for neonatal mass screening in Japan allowed earlier detection of MCADD in the asymptomatic/presymptomatic stage. Subsequent prophylactic management for those children was conducted in a more appropriate and timely manner during metabolic stress such as fever, viral infection and other medical procedures.

Fourteen mutations were identified in 30 independent alleles including seven novel mutations. The amino acids affected by the novel missense mutations (G46D, Q116L, G337E and K395R) are highly conserved among different species (*Pan Troglodytes*, *Rattus norvegicus*, *Xenopus laevis* and *Danio rerio*), suggesting that these amino acids play an important role in medium acyl-CoA dehydrogenase activity. There are also splice site alterations such as IVS3+2T>C, IVS3+5G>A and IVS4+1G>A positioned at a 5′ donor splice site. Shapiro and Senapathy 5′ splice site scores [23] of altered sites changed from 76.4 to 58.6 for IVS3+2T>C, from 76.4 to 62.4 for IVS3+5G>A, and from 86.3 to 68.1 for IVS4+1G>A, respectively, suggesting that these changes are likely responsible for aberrant mRNA splicing. It is reported that point mutations in donor splice site produced exon skipping or aberrant 5′ donor splice site activation [24]. Since these changes likely resulted in aberrant splicing and premature truncation, non-sense mediated mRNA decay [25] or translation into shorter proteins with unlikely residual activity would result.

Most of the mutations detected in Japanese patients were unique, but Q20R, R28C, R256S and c.449–452delCTGA were previously reported in other nationalities [9,22,26,27]. The Japanese patient with compound heterozygous of R28C was one quarter of Caucasian. In contrast, a common missense mutation c.985A>G (80–90%) of Caucasian [8,15,28–30] was not detected in any Japanese patients in this study.

Our study demonstrates that detection in the asymptomatic/presymptomatic stage is essential to achieve favorable outcomes of patients with MCADD. Neonatal mass screening is absolutely a beneficial system to improve the quality of life of patients with MCADD. Genetic background of Japanese patients with MCADD is different from those in Caucasians. It is likely that there is no correlation between genotype and phenotype in Japanese patients with MCADD, and a specific genotype does not predict the clinical outcome.

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今月の主題　周産期の臨床検査

話題

新しい新生児マススクリーニング：タンデムマス法について

山口　清次

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話題

新しい新生児マススクリーニング：タンデムマス法について*

山口清次[1)]

KEYWORDS タンデムマス法，新生児マススクリーニング，有機酸代謝異常症，脂肪酸代謝異常症，拡大スクリーニング
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1．はじめに

新生児マススクリーニングとは，知らずに放置するとやがて重大な健康被害の起こるような代謝疾患を，発症前に発見して障害を予防する事業である．わが国では1977年から全国的に開始され，これまでに約4,300万人の新生児が検査を受け，1万人以上の小児が障害から免れたと考えられている[1,2)]．

新しいスクリーニング検査技術として，最近“タンデムマス法”が開発され普及しつつある[3,4)]．タンデムマス法では，1回の検査で多数の疾患をスクリーニングできるため，小児の障害予防事業を拡大できる技術として普及しつつあるので，現状を紹介したい．

2．これまでの新生児マススクリーニング

わが国ではこれまで，表1に示すような6疾患が対象となっている．方法は，Guthrie法，酵素法，Beutler法，ELISA法(enzyme-linked immunosorbent assay)などが用いられている[2)]．このうち先天性甲状腺機能低下症は，頻度が約3,000人に1人と最も高く，治療薬(レボチロキシンナトリウム水和物)は安価で，早期に治療を開始すれば予後も良く，費用便益の最も良い対象疾患である．副腎過形成症も約1.7万人に1人である．

一方，アミノ酸代謝異常症3疾患では，フェニルケトン尿症の発見頻度は7万人に1人であるが，他の2疾患は数十万人に1人と，極めて稀である．ガラクトース血症は3～4万人に1人発見されるが，大部分は門脈形成異常や原因不明の一過性の症例が占め，先天的酵素欠損によるガラクトース血症1型の頻度は80万人に1人である．極端に稀な疾患は，マススクリーニング対象疾患として疑問視されることもある．

3．タンデムマス法導入による拡大スクリーニング

タンデムマスは，質量分析計を直列に2台並べた構造の分析機器で，超高感度分析できる機器である．使用する検体はこれまでと同じ血液ろ紙の約3 mmのパンチでよく，1回の分析で20種類以上の疾患を一斉スクリーニングができる．ランニングコストはこれまでとあまり変わらない．分析項目はアミノ酸とアシルカルニチンで，現在対象となっているアミノ酸代謝異常症3疾患の他に，有機酸・脂肪酸代謝異常症なども発見できるようになる[5,6)]．

アミノ酸測定値に関しては，Guthrie法よりも精度がすぐれており，偽陽性，偽陰性も少ない．1回の分析は2分程度であり，1台のタンデムマスで，年間5万検体以上が処理できる．対象疾患が拡大すれば，それだけ発見される患者が増え，障害から救われる小児の数も増える．これを“拡大スクリーニング(expanded screening)”という[7)]．

*：Expanded Newborn Mass Screenig for Inherited Metabolic Disease using Tandem Mass Spectrometry

1）YAMAGUCHI Seiji 島根大学医学部小児科・教授

表1　これまでのマススクリーニングの対象疾患と発見頻度

疾患	頻度	費用便益	検査法
1）フェニルケトン尿症	1：7万	○	Guthrie 法
2）メープルシロップ尿症	1：50万	△	（または，酵素法，HPLC 法）
3）ホモシスチン尿症	1：80万	△	
4）ガラクトース血症(全体)	1：3万*	△	Peigen 法
(1型)	(1：80万)		（または，酵素法，Beutler 法）
(2型)	(1：60万)		
5）先天性甲状腺機能低下症	1：3,000	◎	ELISA 法
6）先天性副腎過形成	1：1.7万	○	

＊：ガラクトース高値の多くは酵素欠損でなく，門脈奇形やシトリン欠損症などの2次性のもので，真の先天性ガラクトース血症は極めて稀である．6疾患全体での発見頻度は約1,600人に1人である．

HPLC：high-performance liquid chromatography，ELISA：enzyme-linked immunosorbent assay.

表2　タンデムマス法で発見できる代謝異常症の概略と診断マーカー

		タンデムマスの対象疾患	主な臨床症状	診断マーカー	頻度**
アミノ酸代謝異常症	●	1）フェニルケトン尿症*	けいれん，発達遅滞	Phe	1：6万
	●	2）メープルシロップ尿症*	多呼吸，アシドーシス	Leu+ILeu，Val	1：156万
	●	3）ホモシスチン尿症*	遅れ，発育異常	Met	1：78万
	●	4）シトルリン血症(1型)	異奮，多呼吸，昏睡	Cit	1：26万
	●	5）アルギニノコハク酸血症	異奮，多呼吸，昏睡	Cit，Cit/Arg，ASA	1：40万
	−	6）シトリン欠損症	一過性乳児肝炎類似症状	Cit，Cit/Ser，Phe，Met	1：8万
有機酸代謝異常症	●	1）メチルマロン酸血症	アシドーシス，遅れ	C3，C3/C2	1：12万
	●	2）プロピオン酸血症	アシドーシス，遅れ	C3，C3/C2	1：5万
	●	3）イソ吉草酸血症	アシドーシス，体臭	C5	1：52万
	●	4）メチルクロトニルグリシン尿症	筋緊張低下，ライ症候群	C5-OH	1：16万
	●	5）ヒドロキシメチルグルタル酸血症	重症低血糖，発達遅滞	C5-OH	−
	●	6）複合カルボキシラーゼ欠損症	湿疹，乳酸アシドーシス	C5-OH	1：52万
	●	7）グルタル酸血症1型	アテトーゼ，遅れ	C5-DC	1：18万
	−	8）βケトチオラーゼ欠損症	重症ケトアシドーシス発作	C5-OH，C5：1	−
脂肪酸代謝異常症	●	1）MCAD 欠損症	ライ症候群，SIDS	C8	1：10万
	●	2）VLCAD 欠損症	低血糖，筋肉，心障害	C14：1	1：16万
	●	3）三頭酵素欠損症	ライ症候群，SIDS	C16-OH，C18-OH	−
	●	4）CPT1 欠損症	ライ症候群，肝障害	C0/(C16+C18)	1：31万
	−	5）CPT2 欠損症	ライ症候群，筋肉症状	(16+C18：1)/C2，C16	1：26万
	−	6）TRANS 欠損症	ライ症候群，SIDS	(16+C18：1)/C2，C16	−
	−	7）全身性カルニチン欠乏症	ライ症候群，SIDS	C0(低下)	1：26万
	−	8）グルタル酸血症2型	ライ症候群，低血糖	C8，C10，C12など	1：31万

●＝1次対象疾患(16疾患)，＊：現行マススクリーニングの対象疾患，新～乳：新生児から乳児期，＊＊：2011年までのタンデムマス試験研究のデータ．

SIDS：sudden infant death syndrome(乳幼児突然死症候群)，MCAD：medium-chain acyl-CoA dehydrogenase(中鎖アシル-CoA 脱水素酵素)，VLCAD：very-long-chain acyl-CoA dehydrogenase(極長鎖アシル-CoA 脱水素酵素)，CPT：carnitine palmitoyltransferase(カルニチンパルミトイルトランスフェラーゼ)，SCHAD：short-chain 3-hydroxyacyl-CoA dehydrogenase(短鎖3-ヒドロキシアシル-CoA 脱水素酵素)．

タンデムマス法で発見される疾患と診断マーカーを表2にリストしている．理論的には20数種類の疾患が発見できるが，現時点で，見逃しが極めて少なく，発見すれば治療効果が期待できる16疾患を“1次対象疾患”としている．一方，現時点では，見逃し例が相当数ありうる疾患や，治療効果が十分に確認されてない疾患は“2次対象疾患”として，引き続き検討するとしている[8,9]．

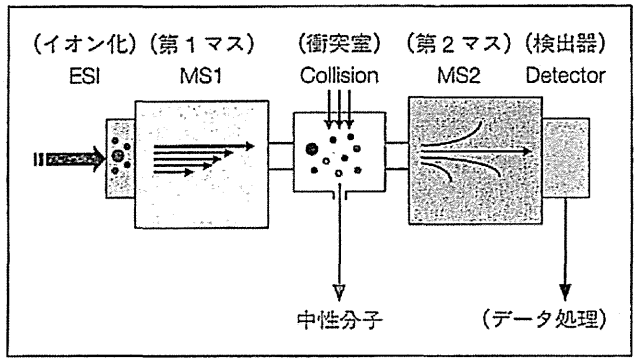

図1　タンデムマス法の原理
ESI：エレクトロスプレーイオン化，MS1：第1マス室(プレカーサーイオン測定)，衝突室でアルゴンガスの粒子によって断片化，MS2：第2マス室(プロダクトイオン測定)．アシルカルニチンはMS1でのプレカーサーイオンをスキャンすることによって測定される．アミノ酸は，プレカーサーイオンとプロダクトイオンの質量数が一定の差を持っているときに，そのプレカーサーイオンが測定される．

4．新しい対象疾患と診断マーカー

表2に示すように，新しく加わる対象疾患として，アミノ酸代謝異常症に含まれる尿素回路異常症の他，有機酸・脂肪酸代謝異常症がある．尿素回路異常症はアミノ酸から生じるアンモニアの処理ができないために高アンモニア血症を起こす．有機酸代謝異常症は，アミノ酸の中間代謝過程の酵素障害によって有機酸が体内に蓄積する．脂肪酸代謝異常症は，β酸化系に障害があるため，ブドウ糖からのエネルギー供給が低下したとき，エネルギー産生不全におちいる．

これらの臨床症状は，生後数日からみられる哺乳低下，多呼吸，意識障害，あるいは感染などを契機に急性発症する急性脳症，突然死などである．一部の疾患では，生後数か月頃から徐々に発達障害が進行するものもある[10]．

診断マーカーは，アミノ酸代謝異常症と尿素回路異常症ではアミノ酸，有機酸・脂肪酸代謝異常症ではアシルカルニチンである．

アミノ酸自動分析計に比べると，タンデムマス法で測定できるアミノ酸は限られているので，スクリーニング対象疾患も限られる．またアシルカルニチン分析では，アシル基を反映する質量数だけの情報となるので，異性体の鑑別はできない．

5．タンデムマス法の概略

タンデムマスは2つの質量分析計が直列に並んだ構造を持つ．図1に示すように，エレクトロスプレーイオン化法(electrospray ionization；ESI)でイオン化された試料が第1質量分析室(第1マス)で質量分析される．この時測定された粒子をプレカーサーイオン(親イオン)という．続いて衝突室でアルゴンガスの粒子に衝突して，粒子は一定の法則で断片化される．これをプロダクトイオン(断片)といい，第2マスで質量分析される[11~13]．

アシルカルニチンはm/z 85の断片をもつことを利用して，第2マスにm/z 85の断片が入ると，第1マスでの親イオンを測定する．これを“ペアレントイオンスキャン法”という．

一方，アミノ酸の場合，衝突室で断片化されたとき中性分子が発生し第2マスには到達できず，ここで失われる．この中性分子は一定の法則に従って生成され，アミノ酸ごとに特定の質量数である．例えば，Val，Leu，Met，Pheなど多くのアミノ酸では質量数46(非誘導体化法の場合)の中性分子が失われる．このことを利用して第1マスで測定した粒子の質量数と第2マスで測定した質量数の差が一定の数(多くは46)のとき，第1マスで測定した質量数の粒子のイオン強度を測定してアミノ酸が測定される．これを“ニュートラルロススキャン法”という．

親イオンをすべてスキャンする方法(スキャン法)とあらかじめ目的とする物質の親イオンの質量数を設定してより高感度に分析する方法(multiple reaction monitoring；MRM)とがある．MRM法では不要なイオンは測定しないため測定感度は良い．一方スキャン法ではアシルカルニチン全体のプロフィールをみることができる．

6．検体の前処理

検体前処理として，“誘導体化法”と“非誘導体化法”とがある[14,15]．その比較を表3に示した．図2に示すように，非誘導体化法は極めて簡便であり，マススクリーニングのような多数検体をスクリーニングするには適している．人件費も節約できる．一方，血液ろ紙の抽出液をそのまま分析するため，誘導体化法に比べ感度の高い機種が要求され，より高額な機器を必要とするため，いまだに誘導体化法を採用している施設は多い．しかし最近安価で高感度の機器が開発されたため，今後は“非誘導体化法”が主流になるであろう．