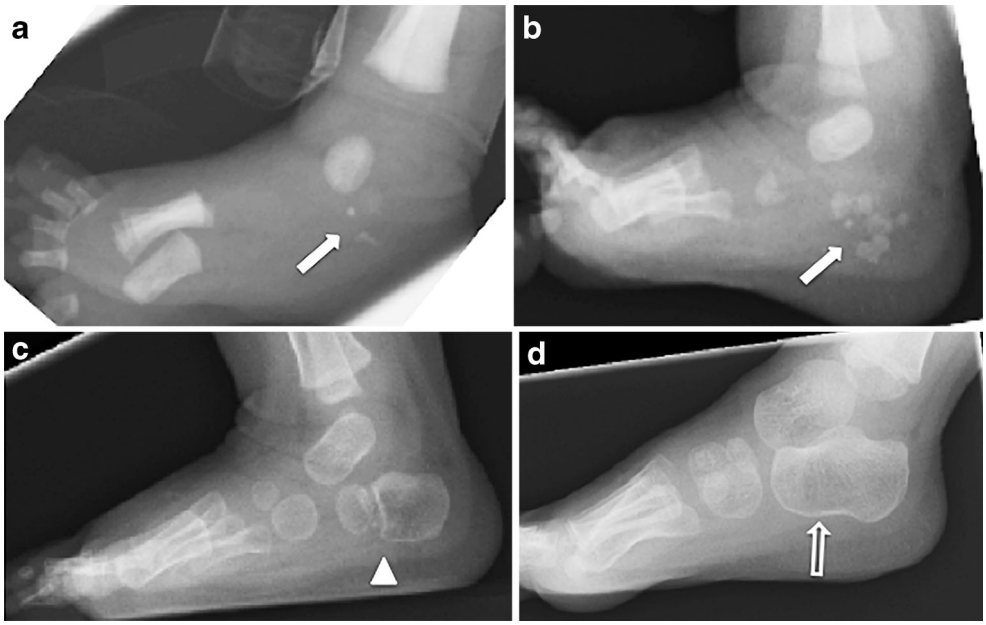

Fig. 1 Lateral foot radiographs in a boy with fibrodysplasia ossificans progressiva (case 1 in Table 1). Sequential radiographs at 1 months of age (**a**), 3 months of age (**b**), 11 months of age (**c**), and 3 years of age (**d**) demonstrate punctate multiple ossifications in early infancy (*solid arrows*), double ossification centers with a cleft separating the anterior third from the posterior two-thirds of the calcaneus (*arrowhead*), and normal calcaneal configuration after complete fusion of the ossification centers (*open arrow*)

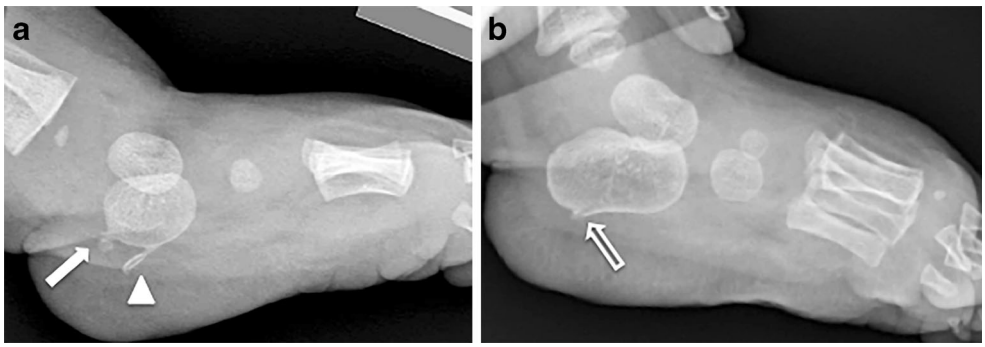

Fig. 2 Lateral foot radiographs in a boy with fibrodysplasia ossificans progressiva (case 2 in Table 1). Sequential radiographs at 2 months of age (**a**) and 12 months of age (**b**) show a small posterior ossification (*solid arrow*) and posteriorly pedunculated calcaneal spur (*arrowhead*), which became smaller in size with age (*open arrow*)

aspect of the anterior calcaneal ossification in infancy. They became sessile and projected inferiorly with age. In patient 2, the spur became smaller in size with age.

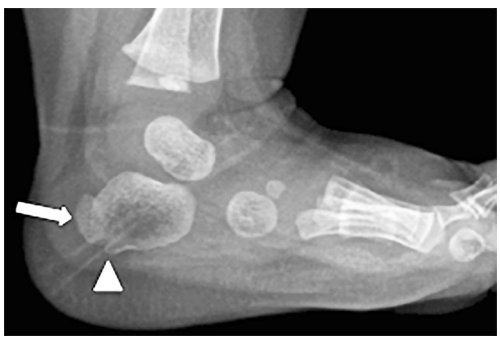

Fig. 3 Lateral foot radiograph in a girl with fibrodysplasia ossificans progressiva (case 3 in Table 1) at 4 months of age demonstrates double ossification centers (*arrow*) and plantar spur (*arrowhead*) of the calcaneus

Discussion

Our findings suggest that abnormal/variant calcaneal ossification, double calcaneal ossification and plantar spurs may be of diagnostic significance in patients with fibrodysplasia ossificans progressiva. These are congenital anomalies, which are perceptible in early infancy and can be a clue to the early diagnosis of this disorder, as are malformations of the great toes, shortening of the thumb and hypertrophy of the posterior element of the cervical spine. This finding is not exclusive of fibrodysplasia ossificans progressiva, as double calcaneal ossifications and plantar spurs are also described as rare developmental variations in normal children [6–10].

The normal double calcaneal ossification is sometimes referred to as bifid os calcis [8–10]. This variation has been reported in children with mild foot deformities, such as pes planovalgus, metatarsus varus and mild talipes equinovalgus. Bifid os calcis shows complete coalescence and remodeling of

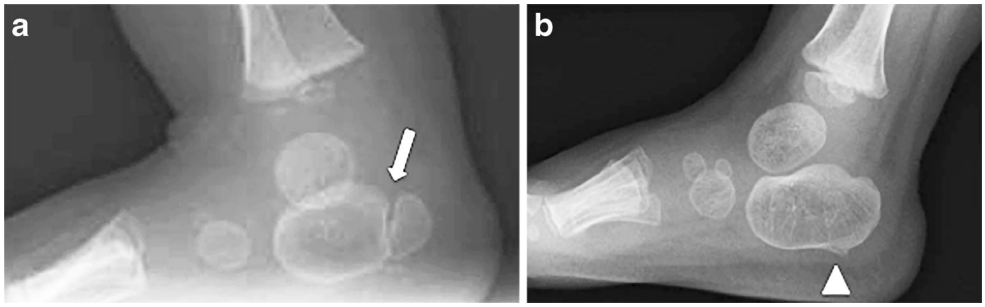

Fig. 4 Lateral foot radiographs in a boy with fibrodysplasia ossificans progressiva (case 4 in Table 1). Sequential radiographs at 10 months of age (**a**) and 22 months of age (**b**) reveal double ossification centers with a cleft separating the anterior two-thirds from the posterior third in the calcaneus (*arrow*) and an inferiorly projected small spur in the plantar calcaneus (*arrowhead*)

two separate ossifications during early childhood and typically shows a cleft separating the anterior third from the posterior two-thirds of the calcaneus. A patient with bifid os calcis reported by Ogden [9] showed punctate ossification centers in the anterior portion. In three children in our series, double calcaneal ossifications were associated with a cleft separating the anterior two-thirds from the posterior third of the calcaneus. However, the manifestation in the remaining one was similar to that in Ogden's case.

A duplicate/triplicate calcaneus was observed in specific skeletal dysplasias, including chondrodysplasia punctata, thanatophoric dysplasia and short rib polydactyly syndromes [11]. Double calcaneal ossifications with a cleft separating the anterior two-thirds from the posterior third of the calcaneus resemble those commonly seen in an infant with Larsen syndrome. Larsen syndrome is caused by heterozygous mutations in filamin B gene (*FLNB*). Filamen B is a cytoskeletal protein involved in a multicellular process [12]. Zheng et al. [13] demonstrated that *FLNB* mutant mice display ectopic mineralization in various cartilaginous elements, including carpal and tarsal bones, and this mutant phenotype is rescued by removing Runx2 through TGFβ-Smad pathway. Overexpression of the R206H mutant ACVR1, on the other hand, enhances Smad1/5 signaling. Molecular interactions between filamin B and Smad signaling in skeletal morphogenesis may lead to similar phenotypes of ossifications in the calcaneal region in Larsen syndrome and fibrodysplasia ossificans progressiva.

The normal plantar calcaneal spur is seen at the posterior two-thirds of the bone, tends to be bilateral and symmetrical, may point anteriorly, posteriorly or inferiorly, and disappears by 1 year of age. The normal calcaneal spur is morphologically indistinguishable from the late manifestation of the calcaneal spur in fibrodysplasia ossificans progressiva. However, the early pedunculated appearance in fibrodysplasia ossificans progressiva is not seen in the normal spur. In addition, the spur in fibrodysplasia ossificans progressiva persists in childhood.

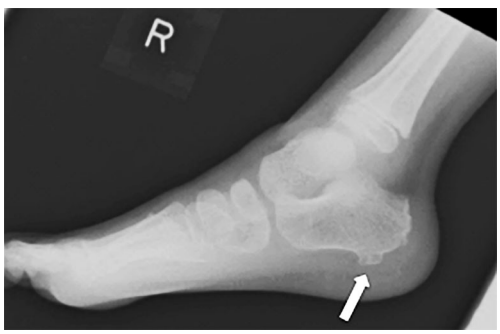

Fig. 5 Lateral foot radiograph in a boy with fibrodysplasia ossificans progressiva (case 8 in Table 1) at 5 years, 10 months of age shows a small spur from the plantar aspect of the posterior calcaneal body (*arrow*)

Conclusion

Double calcaneal ossification centers in early infancy and plantar calcaneal spurs in childhood may be significant radiologic findings useful for early diagnosis of fibrodysplasia ossificans progressiva.

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Compliance with ethical standards

Conflicts of interest None

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Frequency of malformed infants in a tertiary center in Hokkaido, Japan over a period of 10 years

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Abstract

Aim: This retrospective study was performed to determine the frequency of malformed infants born at a tertiary center in Hokkaido, Japan. The accuracy of prenatal diagnosis rates was also investigated.

Methods: An observational study was performed using data of 1509 and 1743 newborn infants at a single center during two study periods, 2005–2009 (first) and 2010–2014 (second), respectively. Cases including minor anomalies (accessory auricle, nevus and fistula auris congenita) were not included.

Results: In total, 274 and 569 malformations were identified in 191 and 337 newborn infants in the first and second study periods, respectively. The number of malformed infants increased significantly over time (13% [191/1509] vs 19% [337/1743], respectively; $P < 0.001$), mainly as a result of an increase in cases of congenital heart disease (CHD), from 59 to 141 (31% [59/191] vs 42% [141/337] of all malformed infants in the first and second periods, respectively). The overall accurate prenatal diagnosis rate improved over time from 47% (128/274) to 58% (329/569) because of significant improvements in accurate prenatal diagnosis of CHD subtypes (23% [16/70] vs 65% [151/232] in the first and second periods, respectively, $P < 0.0001$).

Conclusions: The frequency of malformed newborns was higher in the tertiary center than in the general population. The increased number of cases with prenatal suspicion and diagnosis of CHD contributed to the increased frequency of malformed infants during the study period.

Key words: congenital heart disease, congenital malformation, fetal echocardiography, prenatal diagnosis, tertiary center.

Introduction

Ultrasound examination is widely used in obstetric practice in Japan, leading to antenatal suspicion or diagnosis of fetal malformation. Some malformed infants require prompt treatment specific for individual anomalies soon after birth. Mothers carrying fetuses with suspected or diagnosed malformations are frequently referred to tertiary centers for detailed investigation and subsequent care.

Most university hospitals play a role as tertiary centers in the care of pregnant women with complications. Therefore, it is expected that the frequency of malformed newborn infants would be higher among infants born at tertiary centers than among those in the general population. However, the number of women giving birth to malformed infants in tertiary centers in Japan has not been extensively studied.

Hokkaido University Hospital (HUH), located in Sapporo, is a tertiary center for pregnant women with complications, including fetal anomalies requiring pediatric surgery. HUH covers the Sapporo area in Hokkaido, the northernmost and second largest island in Japan, which had a population of 5 467 000 and 39 392 newborn infants in 2011. The Sapporo area has a

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population of 2 000 000 and 17 000 births annually. In October 2008, Sapporo City launched a system called the Sapporo Obstetric System for Emergency Patients (SOS) to shorten the time interval until admission to an appropriate hospital after the occurrence of an obstetric emergency.[1] Six centers in Sapporo, including the HUH, participated in the SOS system and were considered as having a sufficient number of beds available for such emergency cases. However, the neonatal intensive care unit (NICU) at the six centers, especially at the HUH, suffered from chronic shortages of NICU beds available for otherwise healthy premature infants. This suggested an increased number of maternal referrals for fetal malformations to the HUH from community hospitals remote from the Sapporo area.

This study was performed to determine how many malformed infants were born at the HUH during a 10-year study period from 2005 to 2014. Accurate prenatal diagnosis rates according to malformations were also investigated.

Methods

This retrospective observational study was conducted with approval from the institutional review board of Hokkaido University Hospital (016–0053, May 2, 2016).

A total of 3252 infants were born on or after gestational week 22 at the HUH during the 10-year study period (January 1, 2005 to December 31, 2014). The study period was divided into two five-year periods: 2005–2009 and 2010–2014. We identified newborn infants with malformation(s) using the hospital discharge record database. Thus, malformations were defined as those found during the hospital stay after birth at the HUH. The medical charts of each infant with malformation(s) were reviewed in detail.

Classification of malformations

Minor anomalies, such as accessory auricle, nevus and fistula auris congenita, were not included as malformations in this study. Malformations were classified based on phenotypes regardless of chromosomal/genetic abnormalities or syndrome. Congenital heart disease (CHD) was divided into the following subtypes: pulmonary atresia, pulmonary stenosis, pure pulmonary atresia, atrial septal defect (ASD), tetralogy of Fallot (TOF), extreme TOF, aortostenosis, aortic valve stenosis, coarctation of aorta (CoA), CoA complex, aortic arch interruption (AAI), AAI with ventricular septal defect (VSD), transposition of great arteries (TGA), corrected TGA, endocardial cushion defect (ECD), double outlet right ventricle (DORV), total anomalous pulmonary venous connection (TAPVC), single ventricle, hypoplastic left heart syndrome (HLHS), tricuspid atresia, Ebstein's anomaly, truncus arteriosus communis (TAC) and VSD alone accompanied by neither DORV, TOF, TGA nor CoA complex and others. Patent ductus arteriosus alone was not included in the CHD subtypes.

Accurate prenatal diagnoses of malformations were defined as concordant prenatal and postnatal diagnoses. Small for gestational age (SGA) was diagnosed based on normative birthweight for Japanese infants.[2]

Statistical analyses were performed using the JMP8 statistical software package. Differences between the frequencies were analyzed using Fisher's exact test. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

Results

In total, 1509 and 1743 infants were born at the HUH during the first and second study periods, respectively (Fig. 1). Of these, 191 (13%) and 337 (19%) were identified as having at least one malformation, respectively. Thus, the number of infants with malformation(s) increased significantly over time, from 13% (191/1509) in the first period to 19% (337/1743) in the second ($P < 0.001$). These 191 and 337 infants had 274 and 569 malformations, respectively (Fig. 1). In infants with multiple malformations not affected by trisomy 18 and 13, each malformation was treated separately.

Congenital heart disease was the leading malformation, detected in 200 of the 528 malformed infants (38%). CHD infants accounted for 31% (59/191) versus 42% (141/337) ($P = 0.015$) of all malformed infants born during the first and second periods, respectively. As many as 3.9% (59/1509) and 8.1% (141/1743) ($P < 0.001$) of all infants born in the first and second periods had at least one CHD subtype, respectively. A total of 302 CHD subtypes were observed in the 200 CHD infants (Table 1). VSD alone, pulmonary stenosis and DORV were the leading three CHD subtypes (Table 1). However, the accurate prenatal diagnosis rate for each subtype varied markedly from 3.6% for VSD to 100% for defects such as tricuspid atresia, HLHS, Ebstein's anomaly and pure pulmonary atresia. Thus, the accurate prenatal diagnosis rate for VSD alone was low, perhaps because it is a relatively small defect compared with VSD accompanied by other CHD subtypes. Overall, the accurate prenatal diagnosis rate for CHD subtypes

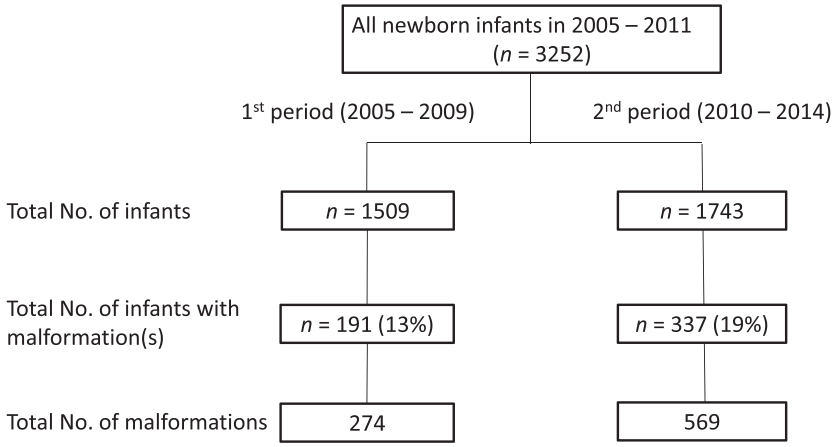

Figure 1 Number of newborn infants with malformations.

Table 1 CHD in 200 infants and rate of accurate prenatal diagnosis of subtypes

Subtype	No. of infants affected	Accurate prenatal diagnosis
Tricuspid atresia	7	7 (100%)
Hypoplastic left heart syndrome	6	6 (100%)
Ebstein's anomaly	5	5 (100%)
Pure pulmonary atresia	4	4 (100%)
Truncus arteriosus communis	1	1 (100%)
Corrected TGA	1	1 (100%)
Endocardial cushion defect	24	20 (83%)
Extreme TOF	6	5 (83%)
Coarctation of aorta complex	6	5 (83%)
TGA	10	8 (80%)
Single ventricle	10	8 (80%)
TAPVC	13	10 (77%)
TOF	14	10 (71%)
AAI with VSD	3	2 (67%)
Pulmonary atresia	13	8 (62%)
Aortic valve stenosis	5	3 (60%)
Double outlet right ventricle	32	19 (59%)
Coarctation of aorta	7	4 (57%)
Pulmonary stenosis	33	16 (48%)
AAI	3	1 (33%)
Atrial septal defect	16	3 (19%)
VSD	56	2 (3.6%)
Others	27	19 (70%)
Overall	302	167 (55%)

All subtypes of CHD seen in 200 infants are listed. Patent ductus arteriosus (PDA) was diagnosed in two newborns, but not included in congenital heart disease (CHD) subtypes in this study. The two with PDA had other anomalies, including ventricular septal defect (VSD) in one and multicystic kidney in the other. AAI, aortic arch interruption; TAPVC, total anomalous pulmonary venous connection; TGA, transposition of great arteries; TOF, tetralogy of Fallot.

was 55% (167/302) (Table 1). CHD subtypes accounted for 26% (70/274) and 41% (232/569) ($P < 0.001$) of all malformations in the first and second periods, respectively.

Other malformations with accurate prenatal diagnosis rates are shown in Table 2. Hydronephrosis (n = 75), hydrocephaly (n = 36), cryptorchidism (n = 26), hypoplasia of the lung (n = 22), corpus callosum agenesis (n = 19), hypospadias (n = 17), congenital diaphragmatic hernia (CDH) (n = 16), duodenal stenosis/atresia (n = 16), cleft lip with cleft palate (n = 16), multicystic kidney (n = 15) and intestinal atresia/stenosis (n = 15) were relatively common malformations at our hospital (Table 2). Among these malformations with higher frequencies, the accurate prenatal diagnosis rate was more than 60% in CDH (94%, 14/15), hypoplasia of the lung (91%, 20/22), hydrocephaly (89%, 32/36), multicystic kidney (87%, 13/15), duodenal stenosis/atresia (81%, 13/16) and corpus callosum agenesis (68%, 13/19).

Both intestinal atresia/stenosis and hypospadias are rare in the general population, with prevalence rates of 8.7 and 5.6 per 10 000 newborn infants in Japan, respectively.[3] In our study, the accurate prenatal diagnosis rate was low for both malformations: 13% (2/15) for intestinal atresia/stenosis and 5.9% (1/17) for hypospadias (Table 2). However, frequencies were very high at our hospital: 15 intestinal atresia/stenosis cases and 17 hypospadias in 3252 infants, corresponding to 46 and 52 per 10 000 infants, respectively. Other malformations/abnormalities were reasons for referral to our hospital in most patients. In the 13 infants who were not prenatally diagnosed with intestinal atresia/stenosis, dilated intestines with (n = 6) or without ascites (n = 5) were observed in 11 fetuses. A cystic abdominal mass was observed in one of the remaining

Table 2 Accurate prenatal diagnosis rates according to malformations in sites other than the heart

	No. of infants affected	Accurate prenatal diagnosis
Polysplenia	9	9 (100%)
Gastroschisis	6	6 (100%)
Congenital cystic adenomatoid malformation	5	5 (100%)
Achondroplasia	4	4 (100%)
Myotonic dystrophy	3	3 (100%)
Campomelic dysplasia	2	2 (100%)
CDH	16	15 (94%)
Hypoplasia of the lung	22	20 (91%)
Hydrocephaly	36	32 (89%)
Spina bifida	9	8 (89%)
Omphalocele	8	7 (88%)
Multicystic kidney	15	13 (87%)
Asplenia	6	5 (83%)
Chiari malformation	6	5 (83%)
Duodenal stenosis/atresia	16	13 (81%)
Osteogenesis imperfecta	7	5 (71%)
Corpus callosum agenesis	19	13 (68%)
Persistent cloaca/bladder exstrophy	3	2 (67%)
Pulmonary sequestration	5	3 (60%)
Renal agenesis/hypoplasia	5	3 (60%)
Ovarian cyst	12	7 (58%)
Hydronephrosis	75	40 (53%)
Cerebellar hypoplasia	6	3 (50%)
Arthrogryposis multiple congenita	2	1 (50%)
Esophageal atresia	9	3 (33%)
Cleft lip with cleft palate	16	5 (31%)
Ectopic gray matter	4	1 (25%)
Cleft lip alone	7	1 (14%)
Intestinal atresia/stenosis	15	2 (13%)
Cryptorchidism	26	2 (7.7%)
Hypospadias	17	1 (5.9%)
Anal atresia	6	0 (0.0%)
Polydactyly (foot)	6	0 (0.0%)
Cleft palate without cleft lip	5	0 (0.0%)
Syndactyly (foot)	4	0 (0.0%)
MMIHS	3	0 (0.0%)
Biliary dilatation	2	0 (0.0%)
Facial cleft	2	0 (0.0%)
Natal teeth	2	0 (0.0%)
Syndactyly (hand)	2	0 (0.0%)

Malformations seen in two or more of the 528 newborns. CDH, congenital diaphragmatic hernia; MMIHS, megacystis microcolon intestinal hypoperistalsis syndrome.

two fetuses. In the 16 infants with hypospadias, also not accurately prenatally diagnosed, nine were twins born to nine twin pregnancies (seven monochorionic and two dichorionic), and six of the remaining seven singletons had multiple anomalies other than the hypospadias, including hydrocephaly (with corps callosum agenesis and SGA), SGA (with cleft lip, cleft palate, cerebellar hypoplasia, DORV and trisomy 18), hydronephrosis (with sacrum deformity, left kidney hypoplasia and anal atresia), SGA (with ASD, VSD and cryptorchidism), hydrocephaly (with hydronephrosis, cryptorchidism and Schinzel-Giedion syndrome) and SGA (with cryptorchidism). Thus, twins were more likely to have hypospadias, and fetal growth restriction was likely to occur in fetuses with hypospadias.

The overall accurate prenatal diagnosis rate was 54% (457/843) during the 10-year study period. However, prenatal diagnostic accuracy was significantly higher during the second period than the first (58% [329/569] vs 47% [128/274], respectively; $P = 0.0212$), which can be attributed to improvement in the accuracy of prenatal CHD diagnosis (Table 3). Accuracy in the prenatal diagnosis rate did not change significantly over time for malformations arising from organs/sites other than the heart (Table 3).

There were 20, 10, and one newborn(s) with trisomy 21, trisomy 18 and trisomy 13, respectively, during the study period, of which 15 (75%), nine (90%) and one (100%), respectively, had malformation(s). CHD was seen in as many as nine (45%) and eight (80%) infants with trisomy 21 and trisomy 18, respectively. The number of infants with these anomalies was 11 (0.73%) during the first period and 20 (1.1%) in the second.

Table 3 Change in rate of accurate prenatal diagnosis over time

	First period (2005–2009)	Second period (2010–2014)
Hydrocephaly	83% (15/18)	94% (17/18)
CDH	100% (5/5)	91% (10/11)
Duodenal stenosis/atresia	67% (4/6)	90% (9/10)
Hypoplasia of the lung	100% (6/6)	88% (14/16)
Corpus callosum agenesis	56% (5/9)	80% (8/10)
Multicystic kidney	100% (6/6)	78% (7/9)
Ovarian cyst	40% (2/5)	71% (5/7)
CHD subtypes	23% (16/70)	65% (151/232)*
Hydronephrosis	57% (21/37)	50% (19/38)
Cleft lip with cleft palate	100% (2/2)	21% (3/14)
Cryptorchidism	17% (1/6)	5.0% (1/20)
Intestinal atresia/stenosis	20% (2/10)	0.0% (0/5)
Hypospadias	14% (1/7)	0.0% (0/10)

*$P < 0.05$ versus first period. Malformations seen in 10 or more infants are listed. CDH, congenital diaphragmatic hernia.

Discussion

As expected, the frequency of malformed infants was much higher in our center compared with the general Japanese population in 2011, based on the 2013 Annual Report released by the International Clearinghouse Center for Birth Defect Surveillance and Research (ICBDSR), in which a limited number of malformations or malformation subtypes are listed.[3] With regard to malformations classified in a similar manner to our method and listed in the ICBDSR report, the frequency of most malformations was much higher in infants born at the HUH. For example, the frequency of hydrocephaly was 169.1 per 10 000 births in this study, far exceeding the rate of 7.8 per 10 000 births in the ICBDSR report, representing a 21.7-fold higher prevalence rate at our center than in the general Japanese population. This suggested that the number of mothers referred to us for suspected fetal malformations and other fetal anomalies suggestive of malformations was high among pregnancies at the HUH. This may have reflected the widespread use of ultrasound examination in antenatal care provided at primary/secondary facilities in Hokkaido.

Indeed, the frequency of intestinal atresia/stenosis and hypospadias was much higher at our hospital than in the general Japanese population, despite the fact that these anomalies were rarely prenatally diagnosed (Tables 2, 3).[3] However, as fetuses with intestinal atresia/stenosis were likely to show dilated intestines, they were referred to us, resulting in a higher frequency at our hospital. Fetuses with hypospadias were likely to exhibit other detectable complications/anomalies, such as twin pregnancy and growth restriction, consistent with results of previous studies.[4,5]

Congenital malformations occur in 2.3% of newborns in the United States after the exclusion of fetuses terminated for severe/lethal malformations.[6,7] CHD is the most common malformation, occurring in approximately 0.8%–1.0% of all live born infants.[8–10] Thus, CHD infants accounted for approximately 30–40% of all malformed infants. Indeed, 38% (200/528) of all malformed infants had CHD in this study. In addition, the CHD frequency increased significantly over time during the study period, with rates of 3.9% (59/1509) and 8.1% (141/1743) of all newborns at the HUH during the first and second periods, respectively. This suggested that Hokkaido physicians responsible for antenatal care had increased their attention to CHD over the past 10 years.

Without intervention, some CHDs are lethal and prenatal diagnosis followed by planned delivery and appropriate postnatal care can improve perioperative morbidity.[11–14] In 2010, the cost of testing with fetal echocardiography targeting women with suspected fetal CHD was included in national health insurance coverage. Screening echocardiography of spatiotemporal image correlation was introduced in seven facilities located in Sapporo in 2013.[15,16] In addition, the HUH participated in a multicenter clinical trial of noninvasive prenatal testing in 2013, resulting in the detection of an increased number of women at high risk with respect to fetal anomalies at the HUH.[17] The number of infants with trisomy 21, 18 and 13 was 0.73% (11/1509) during the first period and 1.1% (20/1743) in the second. These cases may have contributed to the markedly higher frequency of CHD at the HUH and to the significantly improved accuracy in the CHD prenatal diagnosis rate (Table 3). As many as 45% (9/20) of infants with trisomy 21 had CHD in this study, consistent with the results of a previous study in which CHD occurred in 44% (323/728) of all registered infants with trisomy 21 in Europe.[18] Japanese law prohibits any induced abortion on and after gestational week 22. Even in women at less than 22 gestational weeks, induced abortion is allowed only in women with maternal health and/or economic problems, but not in women whose reason for induced abortion is 'fetal anomaly'. These cases may also have been associated with the increase in CHD infants at the HUH, despite improved accuracy in the prenatal diagnosis rate for CHD.

The total number of malformed newborns increased over the study period, from 191 during the first period to 337 during the second. This was mainly a result of the increased number of infants with CHD; the net increase in number of infants with CHD was 82, accounting for 56% of the net increase of 146 malformed newborns. This may be attributed to the fact that in Hokkaido, cardiac surgery for CHD newborns was only available at a limited number of tertiary centers, including HUH, but HUH had no predefined systems for maternal referral for fetal malformations from community hospitals and back transport of infants to community hospitals for convalescent care. Most infants with malformations required NICU admission, causing chronic shortages of available beds for otherwise healthy premature infants. The development of new policies regarding the acceptance of maternal referral with suspected fetal malformations, as well as back transport of infants of very low birthweight to community hospitals, is urgently needed.

Conclusion

In conclusion, this study demonstrated that the number of malformed infants increased significantly over the past 10 years, mainly resulting from an increase in the number of infants with CHD. The overall accuracy rate of prenatal diagnosis improved over time because of significant improvement in CHD prenatal diagnosis. This caused chronic bed shortages for otherwise healthy premature infants at our NICU.

Disclosure

None declared.

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ORIGINAL ARTICLE

Criteria for radiologic diagnosis of hypochondroplasia in neonates

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Abstract
Background A radiologic diagnosis of hypochondroplasia is hampered by the absence of age-dependent radiologic criteria, particularly in the neonatal period.
Objective To establish radiologic criteria and scoring system for identifying neonates with fibroblast growth factor receptor 3 (FGFR3)-associated hypochondroplasia.
Materials and methods This retrospective study included 7 hypochondroplastic neonates and 30 controls. All subjects underwent radiologic examination within 28 days after birth. We evaluated parameters reflecting the presence of (1) short ilia, (2) squared ilia, (3) short greater sciatic notch, (4) horizontal acetabula, (5) short femora, (6) broad femora, (7) metaphyseal flaring, (8) lumbosacral interpedicular distance narrowing and (9) ovoid radiolucency of the proximal femora.
Results Only parameters 1, 3, 4, 5 and 6 were statistically different between the two groups. Parameters 3, 5 and 6 did not overlap between the groups, while parameters 1 and 4 did. Based on these results, we propose a scoring system for hypochondroplasia. Two major criteria (parameters 3 and 6) were assigned scores of 2, whereas 4 minor criteria (parameters 1, 4, 5 and 9) were assigned scores of 1. All neonates with hypochondroplasia in our material scored ≥6.
Conclusion Our set of diagnostic radiologic criteria might be useful for early identification of hypochondroplastic neonates.

Keywords Achondroplasia · FGFR3 · Hypochondroplasia · Neonate · Radiography · Radiologic diagnosis · Scoring system

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Introduction

Hypochondroplasia is the mildest form of fibroblast growth factor receptor 3 (FGFR3)-associated skeletal dysplasia with an incidence of about 1 in 50,000 [1]. Affected individuals usually present after 2 years of age and seek medical help at preschool age because of mild body disproportion and short stature. A diagnosis of hypochondroplasia rests on the presence of several distinctive radiologic findings, such as broad long bones, lumbosacral interpedicular distance narrowing, short femoral necks and elongation of the fibula [2, 3]. However, the diagnosis of hypochondroplasia is hampered by the absence of age-dependent radiologic criteria, particularly in the neonatal period. It has been reported that younger affected children are not definitively diagnosed with hypochondroplasia [4].

We previously reported two cases of hypochondroplasia in children with FGFR3 mutations, focusing on prenatal ultrasonography findings in the third trimester and postnatal radiologic findings [5]. These children had short femora with increased biparietal diameter in utero; however, they were not diagnosed with hypochondroplasia in the neonatal period. The final diagnosis was made at the age of 3 years, when they visited our clinic because of short stature. Upon retrospective radiologic review, we learned that the radiologic findings relevant to hypochondroplasia were apparent in the neonatal period and that radiologic diagnosis may have been even easier in the neonatal period than in early childhood. The manifestations related to the ilia and proximal femora were particularly useful. The identification of short, squared ilia with short greater sciatic notches and horizontal acetabula along with the ovoid radiolucency of the proximal femora mimicking that of achondroplasia warranted the diagnosis.

The present study is dedicated to radiologic features in hypochondroplastic neonates with FGFR3 mutations and quantitative measurements that facilitate definitive diagnosis. We propose radiologic criteria for the identification of hypochondroplasia in the neonatal period.

Materials and methods

Subjects included seven hypochondroplasia neonates with FGFR3 mutations, three term neonates with nonsyndromic fetal growth restriction, and 30 term control subjects with available results of radiologic examination within 28 days after birth. All hypochondroplasia neonates underwent radiologic examination in the neonatal period, such as partial skeletal survey or chest and abdominal radiographs, because of short femoral length on fetal ultrasonography or clinically suspected disproportionate micromelia. Control subjects and individuals with nonsyndromic growth restriction were hospitalized from 2010 to 2014 and were born after 36 weeks of gestation. They did not have major congenital anomalies, and they underwent radiologic examination with extension position of hip joint and knee joint because of transient tachypnea of the newborn, meconium aspiration syndrome or suspected neonatal infection. We hypothesized that skeletal changes in the pelvic bones, femora and lumbar spine, which were seen in achondroplasia, were most useful for the diagnosis of hypochondroplasia. Accordingly, we calculated eight parameters and monitored one radiologic sign: (1) ratio of maximal transverse diameter of the ilia to its maximal longitudinal diameter (assessment of short ilia), (2) iliac angle (squared ilia), (3) length of the greater sciatic notches (short greater sciatic notch), (4) acetabular angle (horizontal acetabula), (5) ratio of femoral length (FL) to body length (femoral shortening), (6) ratio of diameter of the femoral mid-shaft to femoral length (broad femora), (7) ratio of width of the distal femoral metaphysis to femoral length (metaphyseal flaring), (8) ratio of interpedicular distance of the L1 vertebra to that of L4 (lumbosacral interpedicular distance narrowing) and (9) presence or absence of ovoid radiolucency of the proximal femora. Measurement procedures are illustrated in Fig. 1.

The open-source OsiriX software dedicated to the analysis of Digital Imaging and Communications in Medicine (DICOM) images (http://homepage.mac.com/rossetantoine/osirix) was

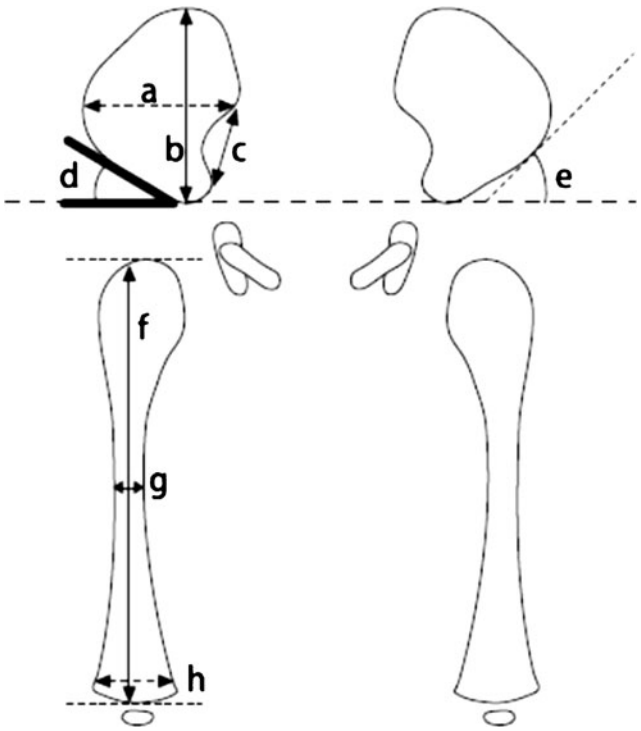

Fig. 1 Diagrams illustrate the measurements based on radiologic findings. The dotted line connects the bottom ends of the ilia: **a** maximal transverse iliac diameter, **b** maximal longitudinal iliac diameter, **c** greater sciatic notch, **d** acetabular roof angle, **e** iliac angle (formed by the tangent line of iliac wing with dotted line), **f** femur length, **g** mid-femur width, **h** maximal distal width of the femur

used for performing measurements. Only radiographs taken with the hip and knee joints extended and without significant joint rotation were analyzed.

Statistical significance of differences between control subjects and hypochondroplasia subjects was analyzed with the Mann–Whitney *U* test. A *P*-value<0.01 was considered significant. All analyses were performed with JMP, version 10.0 (SAS Institute Inc., Cary, NC, USA).

This study was approved by the Institutional Review Board Committee at Niigata University School of Medicine, and informed consent was given by the parents or guardians of the patients with hypochondroplasia.

Results

Clinical manifestations of hypochondroplasia are summarized in Table 1. All the subjects with hypochondroplasia showed low femoral length and biparietal diameter at or above the higher limit of the normal range on prenatal ultrasonography. The results of the Shapiro-Wilk W test showed that all the measurement parameters in the control group followed the Gaussian distribution. The measurement parameters for short ilia, short greater sciatic notch, horizontal acetabula, short femora and broad femora (parameters 1, 3, 4, 5 and 6) were statistically different between the hypochondroplasia and control groups ($P<0.01$), while the remaining parameters were not. Parameters 3, 5 and 6 did not overlap between the 2 groups, while parameters 1 and 4 did (Fig. 2). To distinguish subjects with hypochondroplasia from control subjects, we defined the following cut-off values based on the differences of at least 2 standard deviations from the average values in the control group: >0.80 for parameter 1, <7.5 mm for parameter 3, <22° for parameter 4, <0.14 for parameter 5 and >0.10 for parameter 6. Although assessment of ovoid radiolucency of the proximal femora was somewhat subjective, careful interpretation confirmed its presence in 6 out of 7 children with hypochondroplasia (Fig. 3). There were no abnormalities in other bones.

Based on these results, we defined a tentative scoring system for the diagnosis of hypochondroplasia (Fig. 4). The 2 major criteria (parameters 3 and 6 – short greater sciatic notch and broad femora) were assigned scores of 2. In addition, 4 minor criteria (parameters 1, 4, 5 and 9) were assigned scores of 1 for the following reasons: (a) femoral shortening (parameter 5) was a nonspecific finding; (b) short ilia and acetabular angle (parameters 1 and 4) showed overlaps between the hypochondroplasia neonates and normal controls and (c) the results of the assessment of ovoid radiolucency (parameter 9) were interpreter-dependent. Because all 7 neonates with hypochondroplasia showed combined scores of 6 points or more (Table 2), we presumed that a total score of 6 points or higher warrants thinking about a diagnosis of FGFR3-associated hypochondroplasia. We applied this scoring system to 30 control subjects and the 3 neonates with nonsyndromic growth restriction. The corresponding total scores were less than two in all these cases.

Discussion

It was previously believed that the diagnosis of hypochondroplasia was difficult to establish in infancy. However, recent in utero identification of short femora on prenatal ultrasonography has led to several reports on the early diagnosis of hypochondroplasia [6–10]. It has been found that discrepancy in growth between femoral length and biparietal diameter in the third trimester is highly indicative of this disease [5, 9, 11]. The final diagnosis of hypochondroplasia is established based on the molecular analysis of the FGFR3 gene. This test, however, is relatively expensive and a reliable radiology-based scoring system would be highly beneficial.

Table 1 Genetic and clinical manifestations in 7 children with hypochondroplasia

Child	1	2	3	4	5	6	7
FGFR3 mutation	L324V	N540K	N540K	N540K	S351C	N540K	N540K
Femur length standard deviation score in last trimester	−2.1	−2	-	−3.3	−3.3	−3.5	−2.7
Biparietal diameter standard deviation score in last trimester	0.3	1.3	-	3.3	0.3	3	1.8
Gestational age (weeks) at birth	38	40	38	38	39	38	39
Birth weight (g)	2,780	3,270	2,603	3,102	3,146	2,936	3,228
Birth length (cm)	45.5	49	44.5	49	47	46	45.5
Sex	M	M	F	F	M	F	M
Age at diagnosis[a]	3y 6 m	3y 6 m	1 m	2y	1y 7 m	1 m	1 m

M male, *F* female

[a] The diagnosis was based on the radiologic findings

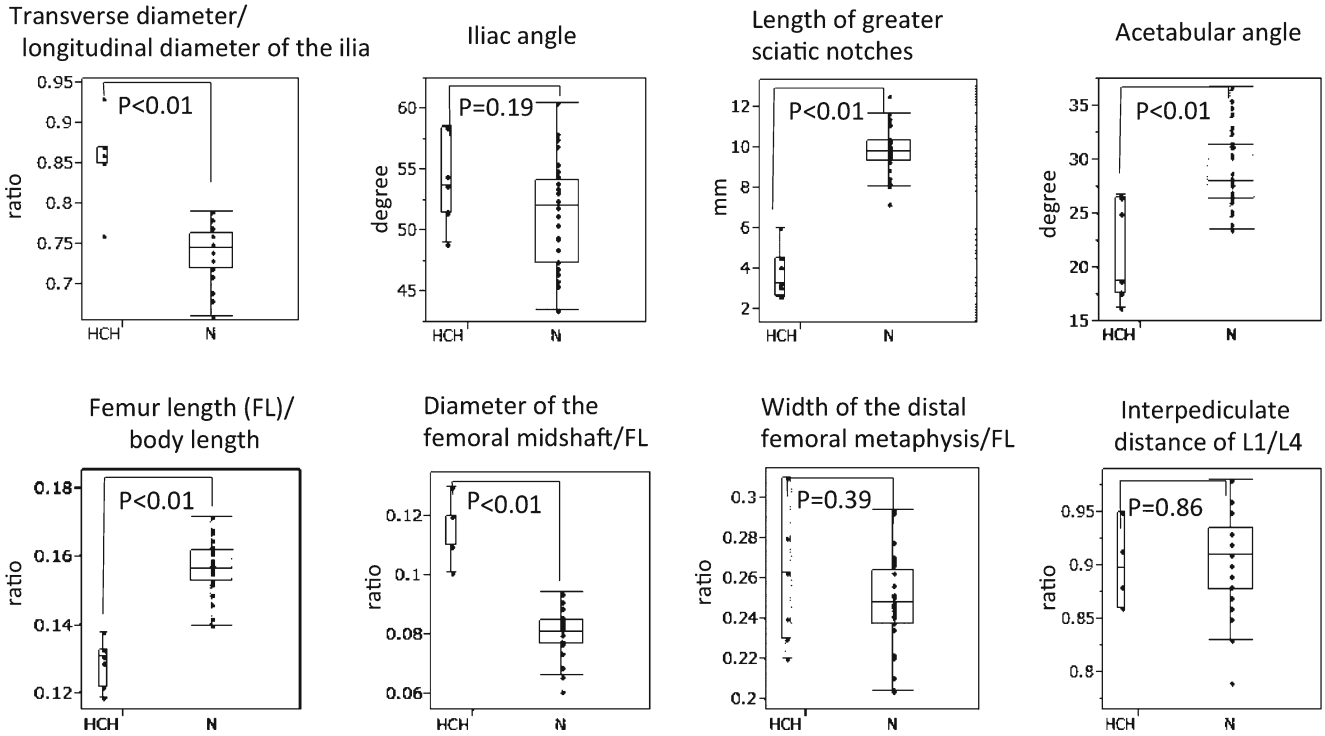

Fig. 2 Results of the measurements in 30 controls and in 7 children with hypochondroplasia. The bottoms and tops of the boxes correspond to the first and third quartiles, respectively, and the horizontal lines inside the boxes indicate the median values. Boxes show as follows: (1) Ratio of maximal transverse diameter to maximal longitudinal diameter of the ilia, (2) iliac angle, (3) length of the greater sciatic notches, (4) acetabular angle, (5) ratio of femoral length to body length, (6) ratio of diameter of the femoral mid-shaft to femoral length, (7) ratio of width of the distal femoral metaphysis to femoral length and (8) ratio of interpediculate distance of L1 to L4. Parameters 1, 3, 4, 5 and 6 were significantly different between the hypochondroplasia and control groups ($P<0.01$). *HCH* hypochondroplasia, *N* control group

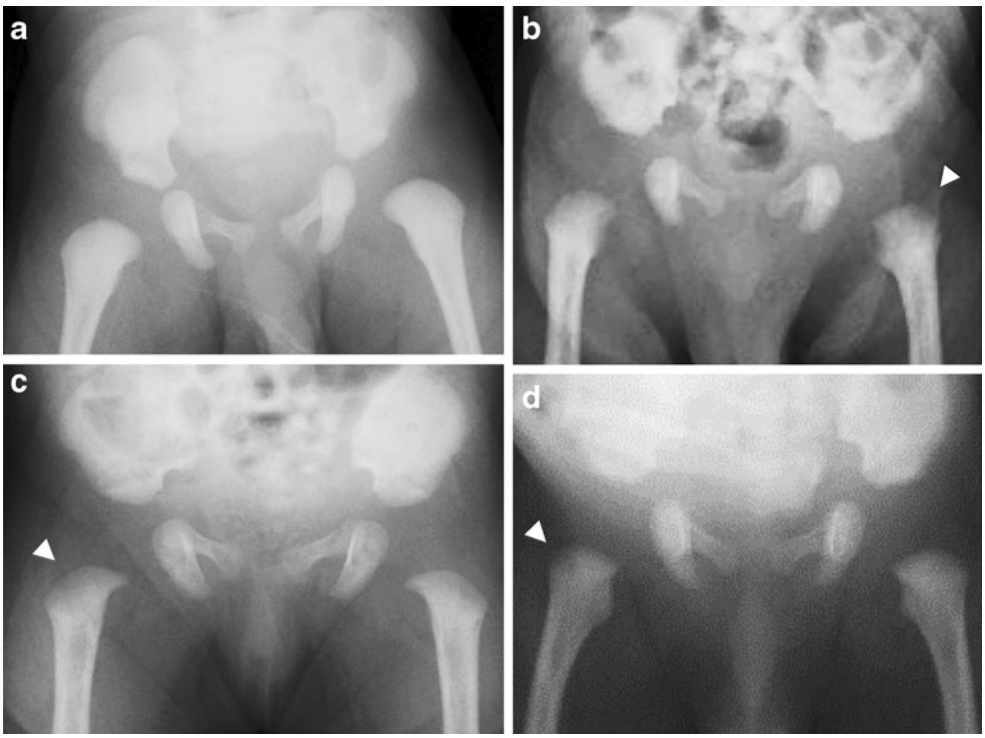

Fig. 3 Ovoid radiolucency of the femoral neck in anteroposterior radiographs. **a** A child in the control group; **b** Child 5 in the hypochondroplasia group (male neonate); **c** Child 6 in the hypochondroplasia group (female neonate); **d** Child 7 in the hypochondroplasia group (male neonate). An ovoid lucency (*arrowheads* in **b-d**) is seen in the femoral neck of the children with hypochodroplasia. All the subjects underwent radiologic examination in the neonatal period.

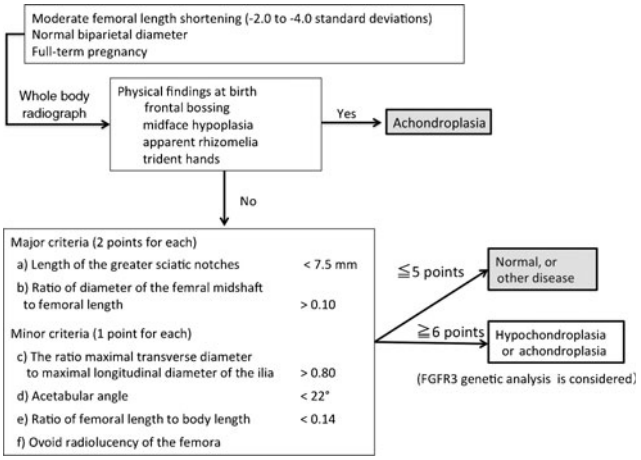

Fig. 4 Proposed flow chart for diagnosis of hypochondroplasia in the neonatal period

In this study, we used radiologic measurements of the ilia and femora to verify that hypochondroplastic neonates had short ilia with short greater sciatic notches and short, broad long bones. Furthermore, ovoid radiolucency of the proximal femora, which reflects the scooped-out appearance of the proximal femoral metaphysis typical of achondroplasia, was always discernible. Horizontal acetabula were also evident, but their presence was inconsistent among the hypochondroplasia neonates. In contrast, although lumbosacral interpedicular distance narrowing is an important diagnostic sign in childhood, it was not useful in our neonatal patients.

Identification of cases with mildly shortened femoral length has become more common with the widespread utilization of fetal ultrasonography. Such cases indicate the presence of mild bone dysplasia exemplified by hypochondroplasia, chromosome disorders such as trisomy 21, and nonsyndromic or syndromic fetal growth retardation (FGR). Although still tentative, our diagnostic criteria might be useful for the differentiation between hypochondroplasia and nonsyndromic growth restriction. Moreover, the radiologic changes in neonates with hypochondroplasia are relatively mild, and their identification may be difficult for nonexperts in bone dysplasias. This emphasizes the potential value of the measurement parameters proposed in the present study.

However, our scoring system is based on nonspecific skeletal changes, such as iliac hypoplasia, a scooped-out appearance of the proximal femora and short, broad femora; thus, it does not enable one to distinguish hypochondroplasia from other skeletal dysplasias, including mild achondroplasia [12]. The final radiologic diagnosis should depend on the overall pattern recognition and other distinctive skeletal changes. For example, cartilage hair hypoplasia causes a diagnostic difficulty in the neonatal period, as does hypochondroplasia [13]. However, mild femoral bowing and round distal femoral epiphyseal ossification warrant a diagnosis of cartilage hair hypoplasia. Molecular diagnoses are essential in difficult cases. To be essential, our scoring system would be utilized as screening for mild neonatal skeletal dysplasias.

The relatively small number of subjects is also a limitation of this study. Furthermore, all measurements were obtained in term neonates; it is currently unknown whether these data are applicable to premature neonates. Finally, correct positioning (extended hip and knee joints without joint rotation) is essential for obtaining interpretable measurements. Further studies in a larger population of hypochondroplasia, including premature neonates, are warranted to validate these criteria for the diagnosis of hypochondroplasia.

Conclusion

We propose a set of diagnostic radiologic criteria that can be useful for early identification of hypochondroplastic neonates.

Table 2 Application of the new scoring system to 7 neonates with hypochondroplasia

Parameter	Child	1	2	3	4	5	6	7
3	Short greater sciatic notches	2	2	2	2	2	2	2
6	Broad femora	2	2	2	2	2	2	2
1	Short ilia	1	1	1	1	1	1	0
4	Horizontal acetabula	1	1	0	1	0	1	0
5	Femoral shortening	1	1	1	1	1	1	1
9	Ovoid radiolucency of the femoral neck	1	0	1	1	1	1	1
Total score		8/8	7/8	7/8	8/8	7/8	8/8	6/8

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Compliance with ethical standards

Conflict of interest None

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ORIGINAL ARTICLE

Fetal cell-free DNA fraction in maternal plasma is affected by fetal trisomy

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The purpose of this noninvasive prenatal testing (NIPT) study was to compare the fetal fraction of singleton gestations by gestational age, maternal characteristics and chromosome-specific aneuploidies as indicated by *z*-scores. This study was a multicenter prospective cohort study. Test data were collected from women who underwent NIPT by the massively parallel sequencing method. We used sequencing-based fetal fraction calculations in which we estimated fetal DNA fraction by simply counting the number of reads aligned within specific autosomal regions and applying a weighting scheme derived from a multivariate model. Relationships between fetal fractions and gestational age, maternal weight and height, and *z*-scores for chromosomes 21, 18 and 13 were assessed. A total of 7740 pregnant women enrolled in the study, of which 6993 met the study criteria. As expected, fetal fraction was inversely correlated with maternal weight ($P<0.001$). The median fetal fraction of samples with euploid result ($n=6850$) and trisomy 21 ($n=70$) were 13.7% and 13.6%, respectively. In contrast, the median fetal fraction values for samples with trisomies 18 ($n=35$) and 13 ($n=9$) were 11.0% and 8.0%, respectively. The fetal fraction of samples with trisomy 21 NIPT result is comparable to that of samples with euploid result. However, the fetal fractions of samples with trisomies 13 and 18 are significantly lower compared with that of euploid result. We conclude that it may make detecting these two trisomies more challenging.
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INTRODUCTION

Noninvasive prenatal testing (NIPT) by massively parallel sequencing has been reported to be highly accurate for the detection of fetal chromosomal aneuploidies.[1–5] This has resulted in widespread adoption of this screening test. Although NIPT has a higher accuracy than conventional prenatal screening method, patients must understand the implications of the results before undergoing testing, including the likelihood of test failure, false positives, false negatives and findings of unclear significance.[3]

In Japan, NIPT for trisomies 21, 18 and 13 was started in April 2013, after receiving approval from the Japan Society of Obstetrics and Gynecology (JSOG) and the Japanese Association of Medical Sciences (JAMS). The initial nationwide trial was conducted by the Japan NIPT consortium.[6] The JAMS has determined that NIPT should be permitted at institutes where appropriate genetic counseling is available.[6,7] The indications for NIPT included a positive maternal serum screen result for an aneuploidy, fetal ultrasound findings indicating an increased risk of aneuploidy, history of a prior pregnancy with a trisomy or maternal age of 35 years or older at the time of delivery.

The placenta releases significant levels of fetal DNA into the maternal circulation, with cell-free fetal DNA fractions reaching levels of 10–20% between 10 and 21 weeks of gestation.[8,9] The cell-free fetal DNA is derived from apoptotic trophoblastic cells in the placenta.[10] Fetal fraction is an important parameter that affects the performance of cell-free fetal DNA-based prenatal tests.[8] Samples with sufficient fetal fractions that pass quality control metrics can provide an accurate assessment of the chromosomes tested.[3,8] Several lines of evidence suggest that the test performance for trisomy 21 is better than trisomies

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18 and 13.[2,8] In contrast, some data show the findings indicating similar detection ability for trisomies 13 and 18 relative to trisomy 21.[9]

Fetal fraction, a key parameter that ensures adequate fetal chromosomal representation, is affected by maternal weight, maternal body mass index (BMI), gestational age and fetal aneuploidy.[3,9,11] Recent reports suggest that fetal fraction correlated positively with gestational age and negatively with maternal weight. Studies that compared fetal fraction among average risk pregnancies in the first trimester did not find it significantly different compared with fetal fractions in high-risk women.[11,12] The purpose of our study was to compare fetal fractions by gestational age, maternal weight and height, BMI, indication of NIPT and *z*-scores for chromosomes 21, 18 and 13. In addition, we examined if a relationship exists between fetal fraction and trisomy involving chromosomes 21, 18 and 13.

MATERIALS AND METHODS

Study population

Pregnant women with high risk for fetal aneuploidy and singleton gestation were enrolled at 10 to 20 weeks of gestation. The high-risk indications included maternal age of $\geqslant 35$ years at the time of delivery, abnormal fetal ultrasound, abnormal serum screen, personal history of a child with aneuploidy or a parent carrying a balanced Robertsonian translocation with an increased risk of trisomy 13 or 21. The study design was approved by all of the hospitals' Institutional Review Board and all women provided informed written consent to participate. NIPT for trisomies 21, 18 and 13 using cell-free DNA in maternal plasma was performed among high-risk pregnant women who requested testing at institutions authorized by the JAMS between April 2013 and March 2014.[6] The details of the study protocol, including the recruitment of high-risk pregnant women who requested testing, are provided on the internet (http://www.nipt.jp/).

Sample collection and preparation

Blood samples (20 ml) were collected from the pregnant women at each institution and were sent to Sequenom Laboratories (San Diego, CA, USA) for MaterniT21 Plus tests within 7 days of collection. If the results were positive, then either amniocentesis or chorionic villus sampling was performed for conventional karyotyping. Exclusion criteria included cases with missing information about maternal characteristics, multiple gestation or fetal demise before NIPT.

Test methods

Cell-free maternal plasma DNA extracted from each sample was subjected to library preparation and massively parallel sequencing using Illumina HiSeq 2000 (Illumina, San Diego, CA, USA) as described earlier.[2,11] SeqFF method is a multivariate regression model that determines fetal DNA fraction.[12] In brief, we used sequencing-based fetal fraction calculations in which we estimated fetal DNA fraction by simply counting the number of reads aligned within specific autosomal regions and applying a weighting scheme derived from a multivariate model. The response variable could be any quantitative metric that reflects fetal DNA fraction. For SeqFF, chromosome Y was chosen as this will allow for the direct comparison of fetal DNA fraction from sequence data rather than a secondary assay. The predictor variables were the aggregated normalized counts of single-end sequence reads aligned to 50 kb contiguously partitioned regions of the human reference genome (hg19). As the magnitude of copy number variation can also be used to estimate fetal DNA fraction, bins located on chromosomes 13, 18, 21, X and Y are excluded from the SeqFF method to avoid issues of model overfitting and circular evidence.[12]

The sequencing data were used to calculate *z*-score, which are robust estimates of normalized chromosomal representation compared with a euploid genome. All samples were required to meet the quality control criteria, including a minimum fetal fraction. *Z*-scores of 3 or above were considered to be indicative of trisomy 21, and *z*-scores 3.95 or above were considered to be indicative of trisomies 13 or 18.

Table 1 Maternal and fetal characteristics of the study population

	Number	*Median*	*Value*
Maternal age (years)	6993	38	36–40
Maternal weight (kg)	6990	52.0	48.0–57.0
Maternal height (cm)	6990	159.0	156.0–163.0
BMI	6990	20.5	19.1–22.2
Gestational weeks (weeks)	6993	13.0	12.0–14.0
Fetal fraction (%)	6991	13.7	10.7–17.9

Abbreviation: BMI, body mass index.
Data are shown as median and interquartile range.

Confirmatory invasive testing

Cases with positive result on NIPT were followed up by villus sampling or amniocentesis to confirm the finding. In cases with intrauterine fetal demise, chorionic villus sampling was performed. Following standard metaphase conversion of cultured fetal cells, conventional karyotyping was performed and at least 20 cells were analyzed. The clinical data, test results and pregnancy outcomes were collected and aggregated every month at the data center of the secretariat. This study is a part of a clinical trial registered with the University Medical Information Network clinical trials registry (UMIN000009338).

Statistical analysis

Statistical methods were used to evaluate the correlation between fetal fraction, maternal characteristics and *z*-scores of chromosomes 21, 18 and 13. Descriptive data of demographic information are presented as median and interquartile range. The measured fetal fraction was represented as square root ($\sqrt{}$) transformed distribution to ensure the normality as described earlier.[13] The association between fetal fraction and maternal weight was calculated by Jonckheere–Terpstra trend test. The differences among levels of variables were compared pairwise using one-way analysis of variance test with *post hoc* Tukey's HSD (honest significant differences) test. *P*-value of $\leqslant 0.05$ indicated a statistically significant difference. Relationships between fetal DNA fraction and *z*-scores in chromosomes 21, 18 and 13 were demonstrated as scatter plots. The statistical analyses, except the trend test, were performed using statistical software package SPSS 22.0 (SPSS, Chicago, IL, USA). The Jonckheere–Terpstra trend test was performed using R version 2.13.0, EZR on R commander version 1.1 designed to add statistical functions frequently used in biostatistics.[14–16]

RESULTS

Of 7740 women who participated in the study, 747 were excluded owing to the lack of details such as maternal and gestational age. Of the 6993 high-risk pregnant women tested in this study, two cases had fetal fraction over 60% and were excluded from the analysis of fetal fraction metrics. Maternal and fetal characteristics of the study population are shown in Table 1 and the frequency distribution of maternal plasma $\sqrt{}$fetal DNA fractions is presented in Figure 1. The $\sqrt{}$fetal fraction has a bell-shaped distribution that peaks between 20 and 40% at 10–20 weeks of gestation.

We examined the relationship between fetal cell-free DNA fraction and gestational age. The median fetal DNA fraction within 10–20 weeks of gestation was 13.7%, with an interquartile range of 10.7–17.9%. More than 99.8% of samples ($n = 6981$) had fetal fraction above the lower acceptable limit for accurate interpretation of fetal aneuploidy. There was no change in fetal DNA fraction from 10 to 20 weeks ($R^2 = 0.02$).

More than 95% of the tests (95.5%, 6677/6993) were performed in women 35 years of age or older with a median age of 38.0 (22–49) years. The median gestational age at the time of testing was 13.0 (10.0–20.2) weeks, the median maternal weight was 52.0 (34.0–115.0) kg and the median BMI was 20.5 (14.5–45.3) $kg\,m^{-2}$ (Table 1). We excluded three of 6993 women because their weight and height data were missing (Table 1). Association between fetal fraction

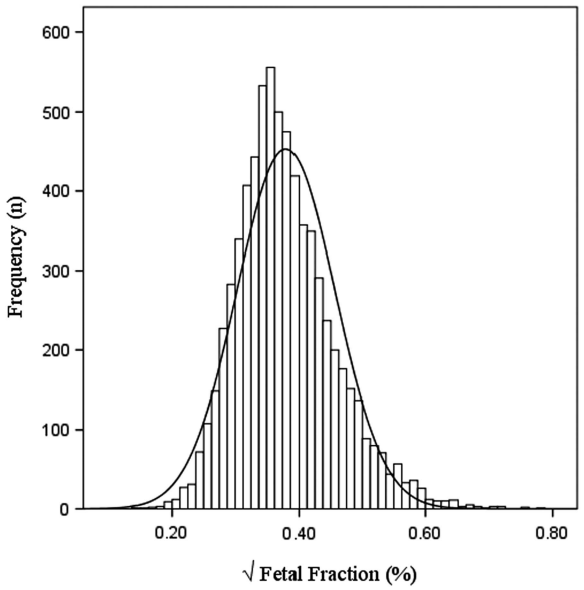

Figure 1 Frequency distribution of square root of fetal fraction in maternal plasma cell-free DNA in the total study of 6991 singleton pregnancies.

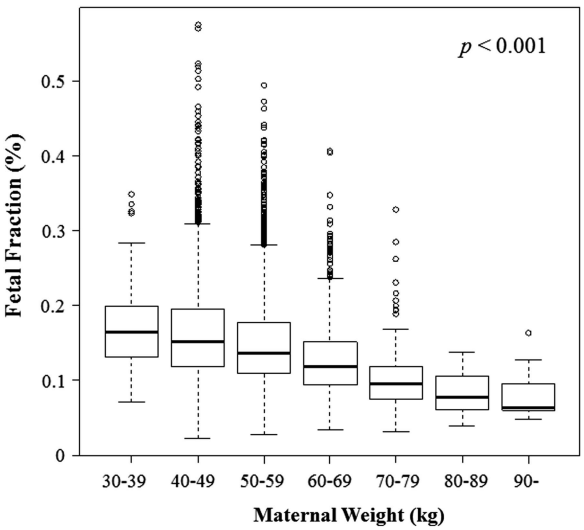

Figure 2 Association between cell-free fetal DNA fraction and maternal weight. It showed an overall trend towards a slight decrease in fetal fraction with increasing maternal weight.

and maternal weight are presented in Figure 2. There was an overall inverse relationship between fetal fraction and maternal weight with a median fetal fraction of 18.1% and 9.6 for maternal weight of <40 and >90 kg, respectively. There was a significant correlation of fetal DNA fraction in early gestational age with maternal weight in 6990 pregnancies, with Jonckheere–Terpstra test (trend test) ($P<0.001$).

In 0.26% of samples (18/6993), NIPT failed because of insufficient fetal DNA or other technical reason, and a 'not reportable' result was issued, of which 16 were retested. Thirteen women found to be negative for fetal aneuploidy, one case had trisomy 18 and two women again received not reportable results. The number of NIPT-positive and -negative cases were 140 and 6851, respectively. Invasive testing using amniocentesis/chrionic villus sampling was performed in 126 NIPT-positive cases, whereas for remaining 14 cases, confirmatory testing could not be performed because of intrauterine fetal death or

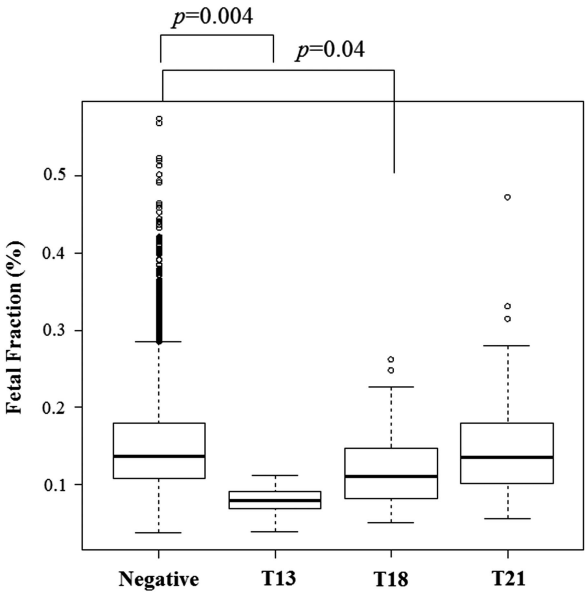

Figure 3 Fetal cell-free DNA fractions in pregnant women carrying fetuses with different trisomies.

other reasons. Conventional karyotyping of amniocentesis/chorionic villus samples confirmed trisomies 21, 18 and 13 in 70, 34 and 9 cases, respectively. The positive predictive value was 95.9% (70/73) for trisomy 21, 81.0% (34/42) for trisomy 18 and 81.8% (9/11) for trisomy 13, respectively. Of the 5483 women who tested NIPT negative for birth outcome was available, only one false-negative case of non-mosaic trisomy 18 was found. This false-negative case had a fetal fraction of 6.06%, and *z*-scores of chromosomes 21, 18 and 13 were -1.867, 2.928 and -1.744, respectively.

Figure 3 depicts fetal cell-free DNA fractions in pregnant women carrying fetuses with different trisomies. Median fetal fraction values for trisomies 21 ($n=70$), 18 ($n=35$) and 13 ($n=9$) were 13.6% (10.2–18.0%), 11.0% (8.1–15.1%) and 8.0% (6.5–10.1%), respectively, although the fetal fraction of NIPT-negative cases ($n=6850$) was 13.7%. In the cases with trisomy 13 or 18, the fetal fractions in maternal plasma were significantly less than that of the NIPT-negative cases by Tukey's HSD (analysis of variance test) analysis ($P=0.004$ and $P=0.04$, respectively). In contrast, no significant differences between NIPT-negative and trisomy 21-positive cases were found ($P=0.9993$).

The relationship of *z*-score for trisomies 21, 18 and 13 and negative samples with fetal DNA fractions is presented in Figure 4. It shows a positive correlation between the *z*-score of trisomies 13, 18 and 21 and fetal fractions. Later gestational age often results in higher positivity rate because of improved classification driven by increased fetal fraction and additional risk factors. Our study, however, did not control positivity rate based on gestational age, instead all the samples between gestational age of 10 and 20 weeks were considered.

DISCUSSION

In this multicenter prospective cohort study, a total of 6993 women among 7740 high-risk women who underwent NIPT were included. Here we show that the fetal fraction in negative and trisomy 21-positive samples by NIPT were not statistically different ($R^2=0.02$). Trisomy 18- or 13-positive samples, by contrast, had

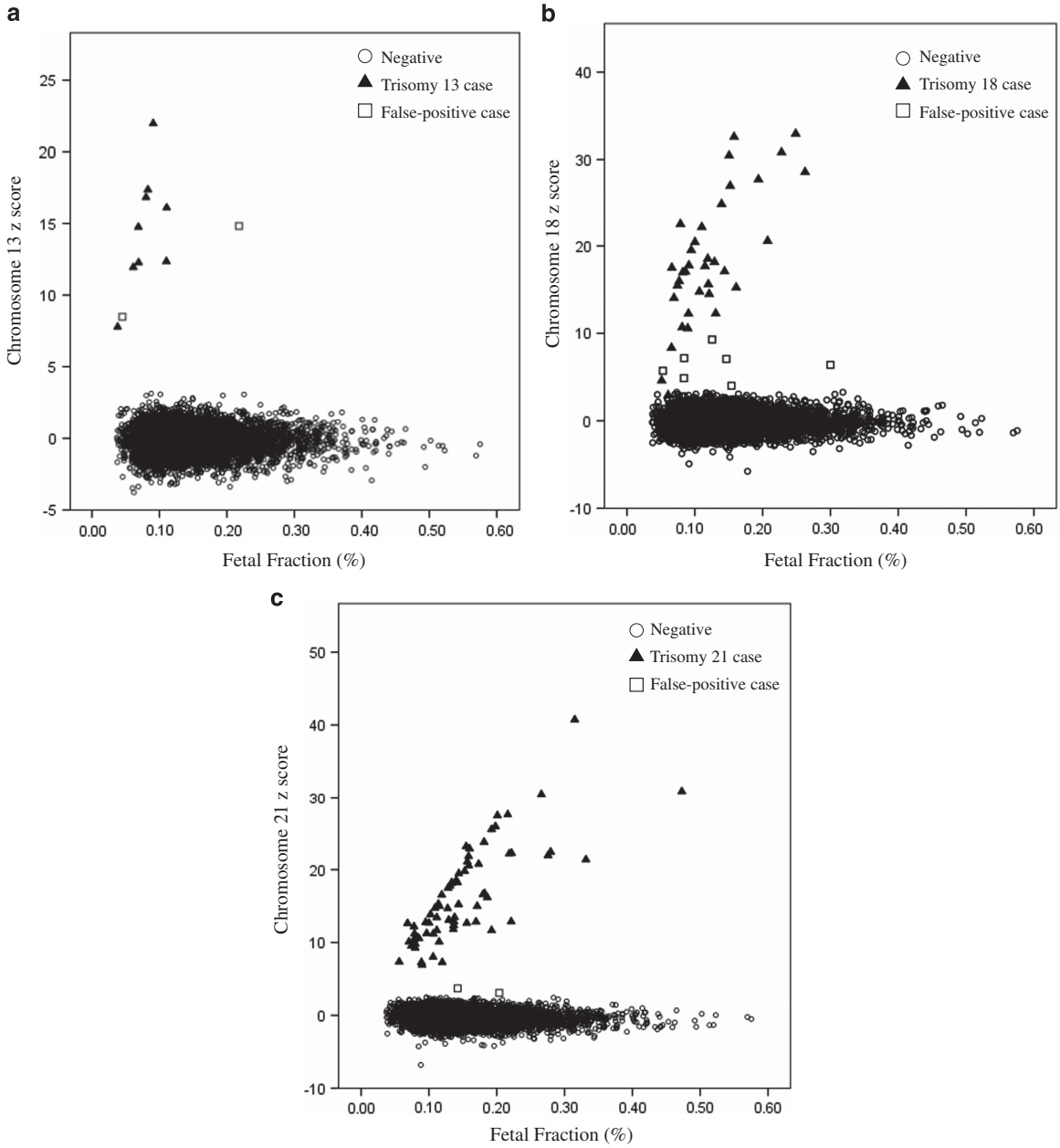

Figure 4 (**a**–**c**) Relationships of the *z*-score with fetal DNA fractions in maternal plasma (**a**, chromosome 13; **b**, chromosome 18; **c**, chromosome 21). Open squares represent false-positive cases ($n=11$; **a**, $n=2$; **b**, $n=7$; **c**, $n=2$).

significantly lower fetal fractions compared with aneuploidy-negative samples. Because fetal fraction is an important quality metric for aneuploidy detection by NIPT, the differential status of specific chromosome aneuploidy may affect the diagnostic accuracy of the test.

In our experience, the fetal DNA fraction between 10 and 20 weeks' gestation showed no significant correlation with gestational age, maternal weight and height, or BMI; in contrast to an earlier report.[17] Shi *et al.*[18] observed an overall positive trend for fetal fractions between the first and second trimester, with 59% of pregnancies showing an increase, 17% showing no change and 24% showing a decrease. However, another study reported that between 10 and 22 weeks gestational age, there was no statistical difference in fetal fraction.[19]

Although circulating DNA in healthy women derives mainly from hematopoietic cells undergoing apoptosis,[20] in obese pregnant women, it partly derives from apoptotic and necrotic cells of adipose and stromal vascular tissues.[21] Our data showed an overall trend towards a slight decrease in fetal fraction in pregnant women who weighed 34 kg (fetal fraction 34.8%) to 115 kg (fetal fraction 6.0%). A similar correlation was observed by Ashoor *et al.*[13] who reported that the median fetal fraction was 11.7% in women who weighed 60 kg, but this decreased to 3.9% in women who weighed 160 kg. They also reported that the estimated proportion with fetal fraction below 4% increased with maternal weight from 0.7% at 60 kg to 7.1% at 100 kg.[13]

In 0.26% of the patients (6975/6993), NIPT failed because of insufficient fetal DNA or other technical reason. This failure rate is lower compared with that previously reported,[2] although all the blood

samples were sent to the United States from Japan. Of the 6993 test-negative patients, birth outcome data was available in 5483 cases, and it identified one false-negative trisomy 18, indicating a false-negative rate of $<0.1\%$.

In the samples positive for trisomies 13 and 18, the fetal fractions were significantly lower compared with that of the NIPT-negative cases ($P=0.004$ and 0.04, respectively), indicating that different fetal aneuploidies have varied effects on the fetal DNA fraction, depending on the affected chromosome, a finding similar to an earlier report.[17] We postulate that smaller placental size and IUGR observed with trisomy 13 and 18 might be contributing to lower observed fetal fraction. It is possible that slow cell cycle speed in trophoblast cells in trisomies 13 and 18 affects the low fetal fraction. In contrast, the trisomy 21-positive samples had fetal fractions similar to that of NIPT-negative samples. Taglauer *et al.*[9] reported that compared with euploid fetuses, those with trisomy 21 have an increased fetal fraction.[9] This apparent higher fetal fractions in samples positive for trisomy 21 may be one of the reasons that test performance for trisomy 21 is better than that of trisomy 13 or 18.[8]

At the end of March 2015, NIPT was carried out at 50 institutions, of which 46 were participants in the Japan NIPT consortium. For further research, assessment of fetal DNA fraction at early gestational age can be important not only in screening for fetal aneuploidy but also for prediction of many pregnancy complications.[22] Our prospective nationwide data of NIPT performance in Japan will be helpful to define the accuracy in a larger scale study. Such a study can detect associations between NIPT result and pregnancy complications (pregnancy-induced hypertension, preeclampsia, fetal growth restriction and preterm birth), and neonatal and long-term prognosis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

Factors affecting parental decisions to terminate pregnancy in the presence of chromosome abnormalities: a Japanese multicenter study†

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ABSTRACT

Objective To investigate the rates of termination of pregnancy (TOP) for fetal chromosomal abnormalities and factors related to such parental decision in Japan.

Methods A multicenter retrospective cohort study of chromosomal abnormalities diagnosed before 22 weeks of gestation between April 2008 and March 2015. The pregnancy outcomes and parental decisions were investigated.

Results Among 931 fetuses with chromosome abnormalities, the total TOP rate was 75.1% (699/931). TOP rates were 89.3% (585/655) in autosomal aneuploidies and 40.8% (51/125) in sex chromosome aneuploidies. Trisomy 21 showed the highest TOP rate (93.8% [390/416]) followed by trisomy 18 (84.5% [163/193]) and trisomy 13 (71.9% [23/32]). Indications for karyotyping were related to a parental decision for TOP ($p < 0.01$): in cases of autosomal aneuploidy, with fetal abnormal ultrasound findings as the reference value, diagnoses made following positive results at non-invasive prenatal testing (adjusted odds ratio [OR]: 13.7, 95% confidence interval [CI] 4.07–45.9) and those because of advanced maternal age (adj. OR 2.91, 95% CI 1.15–7.35) were significantly more frequent.

Conclusions In Japan, pregnancies with fetal trisomy 21 are more likely to result in TOP when diagnosed *in utero* than any other chromosome anomaly. The indications for prenatal karyotyping strongly affect the decision to TOP.

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INTRODUCTION

Fetal karyotyping allows the prenatal diagnosis of fetal chromosome abnormality prior to delivery. In recent years, the proportion of pregnant women ≥35 years of age in Japan has been increasing against decreasing birth rates, leading to an annual increase in the number of fetal karyotyping cases.[1]

The most frequent indication for fetal karyotyping in Japan, where prenatal screening policies have not yet been universally adopted, is advanced maternal age (AMA) defined as age ≥35 years at the expected date of delivery, which accounts for over half of indications.[2] While only 2% of pregnant women choose maternal serum screening (MSS)[1] in Japan, positive results on MSS account for 18% of the indications.[2] In April 2013, non-invasive prenatal testing (NIPT) was introduced in Japan as part of a nationwide clinical research for the detection of trisomy 21, trisomy 18 and trisomy 13.[3] The number of pregnant Japanese women who underwent NIPT has been increasing since then, and about 1% of pregnant women have undergone this testing.[3] However, previous studies have suggested that increased detection rates of fetuses with chromosomal abnormalities by NIPT may lead to an increase in rates of termination of pregnancy (TOP).[4–6]

When fetal chromosome abnormalities are diagnosed by karyotyping, parents become concerned with TOP. Parental decisions to continue or terminate a pregnancy affected with a chromosome abnormality are complicated by the influence of various factors. Previous studies have shown that the TOP rates in cases of chromosomal abnormalities vary greatly by country and ethnicity.[7–10] While recent TOP rates and the factors affecting parental decision for TOP are useful information in the genetic counseling of parents of fetuses with chromosome abnormalities, data for the Japanese population are scarce.

We investigated the TOP rates and factors that contribute to parental decisions regarding a pregnancy after a diagnosis of chromosomal abnormalities before 22 weeks of gestation, which is the legal limit for TOP in Japan.

METHODS

We conducted a multicenter retrospective cohort study at 17 hospitals on pregnant women who underwent fetal karyotyping between April 2008 and March 2015. Of these 17 hospitals, 6 were located in Tokyo and 2 in Osaka; the other 9 hospitals were located in the following cities (1 each): Fukuoka, Isehara, Nagasaki, Nagoya, Nishinomiya, Moroyama, Sapporo, Sendai and Yokohama. Pregnant women with fetuses showing chromosome abnormalities on chorionic villi sampling (CVS) or amniocentesis before 22 weeks of gestations were enrolled and reviewed based on the medical records at each institution. Variants that were common polymorphisms of no known significance were excluded. The legal limit of gestational age for TOP is 22 weeks of gestation in Japan. All of the enrolled women were offered genetic counseling provided by genetic specialists before and after testing. This study was approved by the institutional ethics committee of each institution.

The pregnancy outcomes (TOP, fetal death or live birth) and clinical characteristics, including maternal age, parity, method of conception, number of fetuses, type of diagnostic procedure (amniocentesis or CVS), referral indications for fetal karyotyping and fetal karyotype, were all extracted from medical records at each institution. The indications for karyotyping were classified into five groups: fetal abnormal findings by ultrasound, AMA, increased nuchal translucency (NT) and/or positive result of MSS, positive result of NIPT and others. Because prenatal aneuploidy screening policy is not widely accepted in Japan, NT measurement and/or MSS or amniocentesis for AMA are performed at the request of the parents who chose prenatal screening. NIPT for the detection of trisomy 21, trisomy 18 or trisomy 13 was available for pregnant women at high risk for fetal aneuploidy after April 2013 by request at the patient's expense.[3] In contrast, an ultrasound examination was routinely performed in all pregnant women at each prenatal check-up, with the expenses covered by local governments in most cases. Fetal abnormalities at ultrasound examinations were another major indications for fetal karyotyping. In cases with several indications, the primary indication, that is, the one deemed most influential for the decision, for fetal karyotyping was used for the analysis.

We conducted a descriptive analysis of pregnancy outcomes by fetal karyotype. We also conducted a descriptive analysis of the decision for TOP by autosomal aneuploidy or sex chromosome aneuploidy. Univariate and multivariate logistic regression analyses valuated the association between indications for invasive diagnostic genetic testing and the decision for TOP using abnormal fetal findings by ultrasound as reference group. All multivariate analyses were adjusted for maternal age, parity and method of conception. Statistical analyses were separately performed for autosomal and sex chromosomal aneuploidies. All descriptive and statistical analyses were performed using the STATA version 13 software program (STATA Corp, College Station, TX, USA).

RESULTS

During the 7-year study period, 12,395 pregnant women underwent fetal karyotyping that included 640 (5.2%) cases of CVS and 11,755 (94.8%) cases of amniocentesis before 22 weeks of gestation (Figure 1). Among 978 fetuses (7.9%) diagnosed with chromosome abnormalities, 47 with no pregnancy outcomes were excluded, leaving 931 cases (95.2%) for the analysis.

Table 1 shows the demographics of the pregnant women with a fetal chromosome abnormality. Approximately 75% of these women were ≥35 years old, and over 80% of cases of fetal chromosome abnormalities were diagnosed by amniocentesis. The most frequent indication was increased NT and/or a positive result on MSS (28.8%) and abnormal fetal findings on

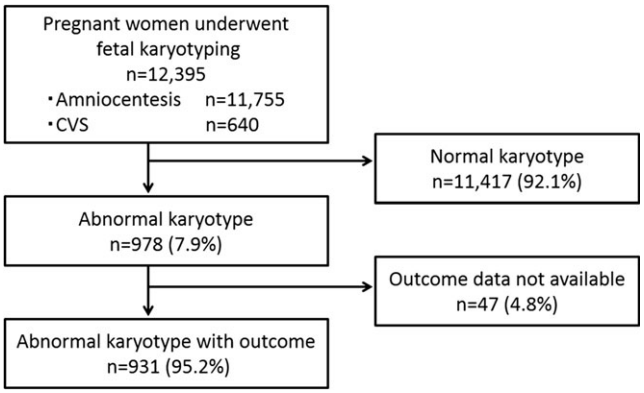

Figure 1 Study subjects between April 2008 and March 2015 in Japan. Among 12,395 pregnant women who underwent fetal karyotyping, 931 with fetuses with abnormal karyotypes were enrolled in the present study

Table 1 Characteristics of pregnant women with a fetal chromosome abnormality

	Number	% of all ($n=931$)
Maternal age, years[a]		
<30	85	9.1%
30–34	143	15.4%
35–39	359	38.6%
≥40	344	36.9%
Having children		
Yes	448	48.1%
No	431	46.3%
Unknown	52	5.6%
Method of conception		
Natural conception	739	79.4%
Assisted reproductive techniques	127	13.6%
Other infertility treatment	47	5.1%
Unknown	18	1.9%
Fetal number		
Singleton	918	98.6%
Twins	13	1.4%
Diagnostic test		
Amniocentesis	786	84.4%
CVS	145	15.6%
Indication for karyotype		
Increased NT and/or positive results on MSS	268	28.8%
Abnormal fetal findings on ultrasound	268	28.8%
Positive result on NIPT	168	18.0%
AMA	158	17.0%
Others[b]	69	7.4%

AMA, advanced maternal age; CVS, chorionic villus sampling; MSS, maternal serum screening; NIPT, non-invasive prenatal testing; NT, nuchal translucency.
[a]Maternal age at the expected date of delivery.
[b]Including carrier of structural chromosome abnormality (39 cases), history of a prior pregnancy with aneuploidy (13 cases), parental anxiety (7 cases), family history of single-gene disorders (8 cases) and history of a prior pregnancy with structural abnormality (2 cases).

ultrasound (28.8%) followed by a positive result on NIPT (18.0%) and AMA (17.0%). Abnormal fetal findings on ultrasound included cystic hygroma, hydrops, cardiac defect, omphalocele, musculoskeletal abnormality, brain abnormality, fetal growth restriction, urogenital abnormality, oligohydramnios, polyhydramnios, facial abnormality and diaphragmatic hernia, in order of frequency. Approximately 80% of pregnancies were achieved by natural conception. Almost half of these women had at least one child already.

The overall distributions of all fetal chromosomal abnormalities and pregnancy outcomes are shown in Table 2. About 70% of the cases were autosomal aneuploidies, including trisomy 21 (44.7%), trisomy 18 (20.7%) and trisomy 13 (3.4%). The TOP rate was 89.3% for autosomal aneuploidy. The highest TOP rate among the autosomal aneuploidies was for trisomy 21 (93.8%), followed by trisomy 18 (84.5%) and trisomy 13 (71.9%). The fetal death and live birth rates for autosomal aneuploidies were 7.3% and 3.4%, respectively. The highest live birth rate among autosomal aneuploidies was for trisomy 13 (9.4%), followed by trisomy 18 (3.6%) and trisomy 21 (2.6%). A univariate analysis showed that trisomy 21 was associated with higher TOP rates than other autosomal aneuploidies ($p<0.01$).

Over half of sex chromosomal aneuploidies were 45,X. For sex chromosomal aneuploidies, the TOP and live birth rates were the same (40.8%). The highest TOP rate among sex chromosomal aneuploidies was for 45,X (47.9%) followed by 47, XYY (42.9%).

Parental decisions for TOP by clinical characteristics are shown in Table 3. The TOP rates varied significantly by indications for karyotyping ($p<0.01$). There were no significant associations among other variables. In cases of autosomal aneuploidies, significantly higher rates of TOP were found for positive results on NIPT (97.6%), AMA (92.9%) and increased NT and/or positive results on MSS (90.0%) ($p<0.01$).

The crude and adjusted odds ratio (aOR) for TOP among indications for karyotyping are shown in Table 4. Regarding the association between indications for karyotyping and decision for TOP of autosomal aneuploidy, with fetal abnormal findings by ultrasound as the reference value, the aOR of positive results on NIPT was 13.7 (95% confidence interval [CI] 4.07–45.9, $p<0.01$), that of AMA was 2.91 (95% CI 1.15–7.35, $p<0.01$) and that of increased NT and/or positive results on MSS was 2.45 (95% CI 1.33–4.50, $p=0.02$). In contrast, regarding the association between indications for karyotyping and decision for TOP of sex chromosome aneuploidy, the aOR of AMA was 0.31 (95% CI 0.09–1.04, $p=0.06$), and that of increased NT and/or positive results on MSS was 0.37 (95% CI 0.14–0.97, $p=0.04$).

DISCUSSION

We presented an analysis of the parental decisions after a prenatal diagnosis of chromosome abnormalities before 22 weeks of gestation in Japan. In our study population, the TOP rate in cases of autosomal aneuploidy was 89.3%, which was consistent with previously published reports in the United States[7,10] and the Netherlands.[11] When compared with the ethnic groups in the United States,[7,10] the TOP rate in the Japanese population in this study was comparable to those of Caucasians and Asians, but higher than those of Hispanics and Filipinos. Such differences may reflect religious and personal preferences in the study populations. The TOP rate for trisomy 21 (93.8%) in the current study was similar to that in other recent population-based or multicenter studies in Australia, China, England and Wales, and Netherlands (92%–94%),[12–15] although it was higher than that in the population-based studies reported from Canada and the United States (67%).[16,17] Among cases of prenatally diagnosed trisomy 21 before 22 weeks of gestation, the live birth rate was only 2.6%. This finding suggests that fetuses with trisomy 21 mostly result in TOP when diagnosed *in utero* during the period in which TOP is allowed.

There are few population-based or multicenter studies examining the TOP rates of trisomy 13 and trisomy 18 in the same study population as trisomy 21. A network of population-based registries in Europe reported that TOP was

Table 2 **Pregnancy outcomes of fetal chromosome abnormality diagnosed before 22 weeks of gestation**

		TOP		Fetal deaths		Live births	
Chromosome abnormality	Number	*n*	%	*n*	%	*n*	%
Autosomal aneuploidy	655	585	89.3%	48	7.3%	22	3.4%
Trisomy 21[a]	416	390	93.8%	15	3.6%	11	2.6%
Trisomy 18[b]	193	163	84.5%	23	11.9%	7	3.6%
Trisomy 13[c]	32	23	71.9%	6	18.7%	3	9.4%
Other trisomy[d]	14	9	64.3%	4	28.6%	1	7.1%
Sex chromosome aneuploidy	125	51	40.8%	23	18.4%	51	40.8%
45,X[e]	73	35	47.9%	21	28.8%	17	23.3%
47,XXY[f]	23	9	39.1%	0	0.0%	14	60.9%
47,XXX[g]	14	1	7.1%	1	7.1%	12	85.7%
47,XYY	7	3	42.9%	0	0.0%	4	57.1%
Other sex chromosome aneuploidy[h]	8	3	37.5%	1	12.5%	4	50.0%
Unbalanced structural rearrangement	74	53	71.6%	4	5.4%	17	23.0%
Balanced structural rearrangement	66	2[i]	3.0%	0	0.0%	64	97.0%
Others[j]	11	8	72.7%	1	9.1%	2	18.2%
Total	931	699	75.1%	76	8.1%	156	14.8%

TOP, termination of the pregnancy.
[a]Includes 4 cases with variant, 3 cases of Robertsonian trisomy 21, 5 cases with balanced structural rearrangement and 1 case of mosaicism.
[b]Includes 2 cases with variant and 2 cases of mosaicism.
[c]Includes 2 cases of mosaicism and 1 case of Robertsonian trisomy 13.
[d]Includes 3 cases of trisomy 9, 2 cases of trisomy 22, 2 cases of mosaicism of trisomy 22, 5 cases of mosaicism of other trisomy and 2 cases of mosaicism of trisomy and double trisomy. Nine cases were diagnosed by amniocentesis, and other 5 cases were diagnosed by CVS.
[e]Includes 16 cases of mosaicism.
[f]Includes 1 case of mosaicism.
[g]Includes 1 case of mosaicism.
[h]Includes 3 cases of XX/XY, 2 cases of XXYY, 2 cases of 45,X/47,XXX and 1 case of XYYY/XYY.
[i]Includes only *de novo* cases.
[j]Includes 7 cases of aneuploidy and structural rearrangement, 2 cases of double trisomy (trisomy 18, XXY) and 2 unknown cases.

more common for trisomy 18 and trisomy 13 than trisomy 21.[9] In our study, however, the TOP rate was highest for trisomy 21. Our study was not designed to explore the variables underlying the parental choices. We hypothesize that viable chromosome abnormalities associated with mental retardation are less acceptable to Japanese parents than those with poorer survival.[18] Another possible explanation is that women may have concerns about ensuring the care of such children after the parents' death and putting a burden on their other children,[19] as the average life expectancy for trisomy 21 individuals has increased to around 60 years.[20] Finally, women may be less familiar with the features of trisomy 13 and trisomy 18, because trisomy 13 and 18 are relatively rare compared with trisomy 21, which is more frequently seen in daily life.

The TOP rate in sex chromosomal aneuploidies was 40.8%. Among sex chromosomal aneuploidies, 45,X led to the highest TOP rate (47.9%), which was in line with the findings of a systematic review of the subject.[21] However, the TOP rates in our study were lower than those in that review, except for those of 47,XYY. This difference may be attributed to the information parents received after the prenatal diagnosis of sex chromosomal aneuploidies. In this study, all of the sessions of genetic counseling before the invasive procedure and after testing with the results of karyotyping were provided by genetic specialists. Some previous studies have included cases that were offered genetic counseling sessions by non-genetic specialists.[21] As reported in the systematic review,[21] genetic counseling by specialists might lead to the continuation of sex chromosomal aneuploidy-affected pregnancies more often than counseling given by non-specialists.

The current study showed that the referral indications for fetal karyotyping contributed to the parental decisions for TOP. Ultrasound examinations are routinely performed in pregnant women at almost every prenatal visit in Japan. As such, for pregnant women referred because of fetal abnormalities found on ultrasound, fetal karyotyping is an unexpected event: such women had declined genetic screening tests. In contrast, parents who choose invasive genetic testing following abnormal results at genetic screening tests may already have a positive attitude towards invasive prenatal testing and possible TOP of the affected pregnancy. Previous studies have shown that the decision to terminate the affected pregnancy was significantly associated with accepting prenatal screening[22] and fetal karyotyping.[23] Our findings suggest that requesting a prenatal genetic screening is more likely to result in a decision for TOP of fetuses with autosomal aneuploidy. In particular, the highest likelihood for TOP was observed in pregnant women referred because of positive results on NIPT.

Table 3 **Parental decision for TOP by clinical characteristics**

	Autosomal aneuploidy			Sex chromosome aneuploidy		
	Terminated			Terminated		
Variables	*n*	(%)	*p*-value	*n*	(%)	*p*-value
Maternal age, years						
<35	93/110	(84.5)		30/63	(47.6)	
≥35	492/545	(90.3)	0.08	21/62	(33.9)	0.12
Having children[a]						
No	265/293	(90.4)		31/75	(41.3)	
Yes	294/333	(88.3)	0.39	20/45	(44.4)	0.74
Method of conception[b]						
Natural conception	463/514	(90.1)		42/103	(40.8)	
Pregnancy after infertility treatment	110/126	(87.3)	0.36	8/21	(38.1)	0.82
Diagnostic test						
CVS	85/99	(85.9)		14/24	(58.3)	
Amniocentesis	500/556	(89.9)	0.23	37/101	(36.6)	0.06
Indication for karyotyping						
Increased NT and/or positive results on MSS	180/200	(90.0)		10/32	(31.2)	
Abnormal fetal findings on ultrasound	152/192	(79.2)		28/51	(54.9)	
AMA	78/84	(92.9)		8/33	(24.2)	
Positive results on NIPT	161/165	(97.6)		1/1[c]	(100)	
Others	14/14	(100)	<0.01	4/8	(50.0)	0.02

AMA, advanced maternal age; CVS, chorionic villus sampling; MSS, maternal serum screening; NIPT, non-invasive prenatal testing; NT, nuchal translucency; TOP, termination of the pregnancy.
[a]No data available for 29 cases of autosomal aneuploidy including 26 terminated cases, and 5 cases of sex chromosome aneuploidy.
[b]No data available for 15 cases of autosomal aneuploidy including 12 terminated cases, and 1 case of terminated sex chromosome aneuploidy.
[c]Positive result of trisomy 18.

NIPT allows for the detection of autosomal aneuploidy at an early gestational age, which may be a reason for the termination of affected pregnancies. However, no significant differences were noted in the TOP rate because of autosomal aneuploidy detected with CVS (earlier detection) versus amniocentesis (later detection) (Table 3). This finding suggests that factors other than early detection may underlie the extremely high rates of TOP among pregnant women with positive results on NIPT.

In cases of sex chromosome aneuploidy, however, pregnant women referred because of AMA and increased NT and/or positive results on MSS were less likely to terminate the affected pregnancy than those referred because of abnormal ultrasound findings. This opposite trend in the decisions

Table 4 **The crude and adjusted odds ratios for TOP among indications for karyotyping**

	Autosomal aneuploidy			Sex chromosome aneuploidy		
Indications	Crude OR (95% CI)	aOR[a] (95% CI)	*p*-value	Crude OR (95% CI)	aOR[a] (95% CI)	*p*-value
Abnormal fetal findings on ultrasound	Ref	Ref		Ref	Ref	
Increased NT and/or positive result on MSS	2.37 (1.33–4.22)	2.45 (1.33–4.50)	0.02	0.37 (0.15–0.95)	0.37 (0.14–0.97)	0.04
AMA	3.42 (1.39–8.42)	2.91 (1.15–7.35)	<0.01	0.26 (0.10–0.69)	0.31 (0.09–1.04)	0.06
Positive result of NIPT	10.6 (3.70–30.3)	13.7 (4.07–45.9)	<0.01	—	—	

AMA, advanced maternal age; aOR, adjusted odds ratio; CI, confidence interval; MSS, maternal serum screening; NIPT, non-invasive prenatal testing; NT, nuchal translucency; OR, odds ratio; TOP, termination of the pregnancy.
[a]Adjusted for maternal age, parity, method of the conception.

compared with autosomal aneuploidy can be explained by prior data indicating that fetal ultrasound abnormalities had a strong influence on the decision for TOP of sex chromosome aneuploidies.[24,25] Therefore, abnormal ultrasound findings may adversely influence the decision to continue the pregnancy.

Several limitations associated with the present study warrant mention. First, as our study was a retrospective review of medical records without any interviews, we were unable to assess the individual and complex psychological factors that influence parental decisions. Second, all of the participating institutions are special in Japan, providing genetic counseling before and after testing by genetic specialists. Therefore, our study is unable to represent the TOP rates among institutions where non-specialists are involved in genetic counseling. Finally, we do not know how many couples with sonographic evidence of fetal anomalies chose TOP without undergoing genetic diagnostic tests.

In conclusion, the TOP rate of trisomy 21 (93.8%) was significantly higher than that of other autosomal aneuploidies. TOP rates are affected by the indication for invasive genetic testing. In particular, the highest likelihoods of TOP were observed for pregnant women referred because of elevated risk of aneuploidies based on genetic screening tests.

WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- The rates of termination of pregnancy (TOP) because of chromosomal abnormalities vary greatly by country and ethnicity, and the termination rate in Japan is unknown.
- Parental decisions to continue or terminate a pregnancy affected with a chromosome abnormality are influenced by various factors.

WHAT DOES THIS STUDY ADD?

- Trisomy 21 showed the highest TOP rate (93.8%), with a live birth rate of 2.6% in Japan for prenatally diagnosed cases.
- Indications for prenatal karyotyping, especially positive results at non-invasive prenatal testing, strongly contribute to TOP when a chromosomal abnormality is found.

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Survey of prenatal testing for genetic disorders in Japan: Recent report

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Abstract

Aim: In order to investigate the current status of prenatal testing for genetic disorders, we conducted a multicenter retrospective questionnaire survey.

Methods: The questionnaire was sent to 105 facilities with genetic counseling systems. The questionnaire consisted of two parts: (i) the number of prenatal tests conducted for genetic disorders from January 2010 to December 2012, whether the laboratory was combined with the counseling facility or separate, the sampling procedure method, the testing results, and the outcomes of the affected fetus in addition to treatment; and (ii) a survey of personal comments regarding prenatal testing for genetic disorders.

Results: We received responses from 69 of the 105 facilities (65.7%), and genetic testing was performed at 26 of these facilities. Nucleic acid sequential testing was performed on 45 disorders and 252 cases during a three-year period. There were 67 cases of affected fetuses. Six cases continued pregnancy and were treated. The comment survey highlighted difficulties in locating a laboratory to assess prenatal samples, as well as inadequate counseling and preparation for genetic disorders.

Conclusions: Our study revealed that a number of prenatal testing for genetic disorders are conducted in Japan; however, it is difficult for counselors to locate a laboratory capable of testing for specific genetic disorders. Inadequate counseling and healthcare providers' lack of knowledge is a current problem. A well-established system of prenatal testing for genetic disorders and the further education of general physicians is required.

Key words: counseling, genetic disorder, prenatal testing.

Introduction

The advances in molecular genetics enable us to analyze gene mutations in patients with genetic disorders. Genetic information remains constant over a lifetime; therefore, it requires careful scrutiny via preclinical and prenatal genetic testing. The Japanese Association of Medical Sciences and the Japan Society of Obstetrics and Gynecology (JSOG) developed guidelines and opinions for appropriate genetic counseling prior to prenatal genetic testing.[2,3] Based on this, pregnant carriers of genetic disorders in the neonatal or childhood periods can obtain adequate genetic counseling before and after prenatal testing for the genetic disorder.

Several methods for the prenatal testing of genetic disorders are available. The majority test for chromosomal abnormalities, such as aneuploidy, by means of a maternal serum blood test, ultrasound examination, and noninvasive prenatal genetic testing.[4] Recent advances in molecular biology have made major contributions to

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prenatal diagnosis. An increasing number of genetic disorders have been identified in the prenatal period using fetal cells derived from invasive testing.[5–9]

In some genetic disorders, such as ornithine transcarbamylase deficiency, prenatal genetic testing is useful for neonatal treatment.[10] However, general obstetricians are not familiar with the requirements for prenatal testing and lack current information regarding genetic testing and neonatal treatment. Additionally, in Japan, private companies that provide DNA testing do not offer the prenatal service, and almost all prenatal molecular testing is performed in non-commercial research laboratories. Therefore, it is difficult to find an appropriate laboratory for the prenatal testing of genetic disorders.

In order to investigate the current status of prenatal testing for genetic disorders, we, as members of the subcommittee in Perinatology Committee of the JSOG, conducted a multicenter retrospective questionnaire survey.

Methods

The questionnaire was sent to 105 facilities, including Miyagi Children's Hospital and 104 other facilities, which belong to Japan's National Liaison Council for Clinical Sections of Medical Genetics and contain genetic counseling systems.

The questionnaires consisted of two parts: (i) the number of prenatal testing for genetic disorders evaluated from January 2010 to December 2012, whether the laboratory that received the samples contained a counseling facility or the testing was outsourced domestically or abroad, the sampling procedure, the testing results and the outcome of the affected fetus and treatment; and (ii) a survey of personal comments for prenatal genetic testing. We allowed for five options and space for a description.

Results

We received responses from 69 of the 105 facilities (65.7%). Prenatal testing for genetic disorders was conducted on 46 disorders and 277 cases during a three-year period. Testing was performed at 26 facilities (Table 1). Nucleic acid sequential testing was performed on 45 disorders and 252 cases. Only gender testing was performed on nine disorders and 22 cases, while only enzymatic activity testing was performed on two disorders and two cases. Genetic testing was not performed for a chromosome abnormality in one case.

The number of prenatal tests conducted for each disorder and the testing laboratory is presented in Table 2. The testing laboratory and the counseling facilities were the same for 32 disorders and 142 cases (56.3%). The testing was outsourced within Japan for 26 disorders and 107 cases (42.5%). Among them, 13 disorders were tested at another facility that responded to the questionnaire. The remaining disorders were assessed at unknown facilities. Genetic testing was conducted abroad for three disorders and three cases (1.2%); however, all three diseases could have been assessed at one of the domestic facilities that responded to the questionnaire.

Table 1 The number of prenatal tests for genetic disorders conducted at each facility during a three-year period

Number of genetic tests	Number of facilities
1–3	12
4–9	7
More than 9	7

Ninety-four genetic tests were conducted at the National Center for Child Health and Development, 64 at Tokyo Women's Medical University Hospital, 16 at Osaka University Hospital, 12 at Tottori University Hospital, 10 at Hokkaido University Hospital, 10 at Hiroshima University Hospital and 10 at Miyagi Children's Hospital.

In regard to the type of sampling procedure, chorionic villi sampling (CVS) was conducted on 203 cases (73%), amniocentesis on 70 cases (25%), and the other four cases (2%) entailed fetal gender testing via maternal blood examination. There were 67 cases of affected fetuses. Among autosomal dominant disorders, four of 12 cases (33.3%) were affected, while 63 of 240 cases (26.2%) were affected by recessive disorders. Two cases were undefined as a result of contamination.

The outcomes of the affected fetuses are presented in Table 3. In the six cases in which the pregnancy was continued, the disorders were treated. One infant affected with the Wiskott-Aldrich syndrome was treated with an unknown medication after birth. In two cases of a female fetus affected with 21-hydroxylase-deficient congenital adrenal hyperplasia (21-OHD CAH), the mothers continued glucocorticoid intake to prevent neonatal masculinization of genitalia. In a case of a male fetus affected with 21-OHD CAH, the mother discontinued glucocorticoid intake after being apprised of the fetal gender. In another case of a female fetus affected with 21-OHD CAH, the prenatal diagnosis suggested an atypical outcome of 'not affected'. Because of the incorrect prenatal diagnosis, the mother discontinued glucocorticoid intake and the infant exhibited masculinization of genitalia at birth. In a case of myotonic dystrophy, prenatal testing showed that the fetus was not affected; however, the infant was floppy, and was diagnosed with congenital myotonic dystrophy.

Table 2 The number of genetic disorders identified and destinations of the testing samples

Disease	Number of genetic tests	Number of cases/facilities (Same)†	Number of cases/facilities (Domestic)‡	Number of cases/facilities (Abroad)§
Neuromuscular Diseases				
Fukuyama-type congenital muscular dystrophy	52	24/4	28/7	ND
Spinal muscular atrophy	46	19/1	27/9	ND
Duchenne muscular dystrophy	38	28/6	10/2	1/1
Myotonic dystrophy	9	1/1	8/5	ND
X-linked hydrocephalus	6	2/2	4/4	ND
Myotubular myopathy	5	3/2	2/2	ND
Ryanodine receptor disorders	5	2/2	3/1	ND
Pelizaeus-Merzbacher disease	4	3/1	1/1	ND
Adrenoleukodystrophy	1	ND	1/1	ND
Lissencephaly	1	ND	1/1	ND
Joubert syndrome	1	ND	1/1	ND
Metabolic Diseases				
Ornithine transcarbamylase deficiency	7	6/3	1/1	ND
Hunter syndrome	6	5/1	1/1	ND
Carbamoyl phosphate synthetase deficiency	4	3/2	1/1	ND
Gaucher disease	4	2/1	2/2	ND
Methylmalonic acidemia	3	3/3	ND	ND
Mucolipidosis II	3	3/2	ND	ND
MCT8 gene disorder	3	ND	3/1	ND
Menkes disease	2	2/1	ND	ND
Krabbe disease	2	2/1	ND	ND
Zellweger syndrome	2	ND	2/2	ND
Pompe disease	1	1/1	ND	ND
Pyruvate dehydrogenase complex deficiency	1	1/1	ND	ND
Glutaric acidemia	1	1/1	ND	ND
Very-long-chain acyl-CoA dehydrogenase deficiency	1	1/1	ND	ND
Trifunctional protein deficiency	1	1/1	ND	ND
Metachromatic leukodystrophy	1	1/1	ND	ND
Tay-Sachs disease	1	1/1	ND	ND
Holocarboxylase synthetase deficiency	1	ND	1/1	ND
Niemann-Pick disease	1	ND	1/1	ND
Endocrine Diseases				
21-Hydroxylase-deficient congenital adrenal hyperplasia	17	13/4	4/3	ND
Skin Diseases				
Xeroderma pigmentosum	3	3/1	ND	ND
Congenital ichthyosis	3	ND	3/3	ND
Bone Diseases				
Hypophosphatasia	3	3/2	ND	ND
Osteogenesis imperfecta II	1	ND	1/1	ND
Achondroplasia	1	1/1	ND	ND
Urinary Tract Disorders				
Lowe syndrome	2	1/1	ND	1/1
Autosomal recessive polycystic kidney	1	1/1	ND	ND
Congenital nephrotic syndrome of the Finnish type	1	ND	1/1	ND
Immunodeficiency Diseases				
X-linked severe combined immunodeficiency	2	2/1	ND	ND
Wiskott-Aldrich syndrome	2	1/1	ND	1/1
Chronic granulomatous disease	1	1/1	ND	ND
Other				
Juvenile polyposis syndrome	1	ND	1/1	ND
Bannayan-Riley-Ruvalcaba syndrome	1	ND	1/1	ND

†Same: the laboratory that conducted the genetic testing was the same facility. ‡Domestic: the laboratory that conducted the genetic testing was another domestic facility (in Japan). §Abroad: the laboratory that conducted the genetic testing was abroad (outside Japan). ND, no data.

Table 3 Outcomes of affected fetuses

Outcome of affected fetuses	Number
Termination of pregnancy	57 (85%)
Continuation of pregnancy	6 (9%)
Unknown	4 (6%)

The percentage of respondents who provided comments is presented in Table 4. Representative descriptions included: 'It is necessary to locate an appropriate laboratory following each instance of genetic counseling, and the requirements for genetic testing differ for each laboratory'; and 'The incomplete system for genetic testing in domestic laboratories forces an increasing number of samples from patients to be sent abroad'.

Discussion

In this report, we presented the current status of prenatal testing for genetic disorders in Japan. Forty-five disorders and 252 cases of prenatal genetic nuclear acid sequencing testing were conducted during a three-year period. To the best of our knowledge, this is the first nationwide report regarding prenatal testing for genetic disorders in Japan. However, this survey had some limitations.

Any single facility could not conduct all types of genetic testing. The testable genetic disorders differed at each facility. Therefore, it was difficult to ascertain how many samples were submitted to outsourced laboratories. In this survey, 13 genetic disorders could not be assessed at the responding facilities. This indicated that a facility not surveyed or that did not respond to the questionnaire actually conducted a number of prenatal tests for genetic disorders.

Table 4 Comments for prenatal testing for genetic disorders

Comments	Agreement† (%)
There are no commercial laboratories to treat prenatal genetic samples in Japan.	78.3
Patient consultation was too late for appropriate evaluation (e.g. after pregnancy).	58.0
Case lacks genetic testing of the proband.	52.2
Approval by ethical review board is required in each case.	37.7
Genetic counseling was inadequate.	20.3

†Agreement denotes the percentage of respondents who made the comment.

In the comment survey for prenatal genetic testing, 78% of responding facilities advised that no commercial laboratory was available to assess prenatal genetic samples. This might indicate frustration with suboptimal systems for the prenatal testing of genetic disorders in Japan. Some respondents were unsure of the accuracy of the testing, and misinterpretation could lead to an inappropriate choice. Furthermore, the individuals in charge of the prenatal testing carried a heavy responsibility in terms of the results. It is difficult for counselors to obtain appropriate prenatal testing in Japan. Even if a domestic laboratory could have conducted the prenatal testing for a genetic disorder, the counselors may have thought that it would be easier to send the samples abroad. Testing abroad may often be an expensive and time-consuming process; thus, a well-established system of prenatal genetic testing within Japan is needed.

In the comment survey, 58% of the responding facilities stated that the consultation for prenatal testing was too late for appropriate evaluation (e.g. after establishment of next pregnancy); and 52% of the respondents commented that some cases lacked genetic testing of the proband. Furthermore, some respondents also noted that previous counseling was inadequate, which impacted the counseling quality for subsequent pregnancy. These indicated that general physicians and patients were unfamiliar with the preparation required for appropriate genetic testing in subsequent pregnancy. Therefore, education of general physicians regarding prenatal testing for genetic disorders would be beneficial.

In conclusion, our study revealed that a number of prenatal testing for genetic disorders were conducted in Japan; however, it was difficult for counselors to locate an appropriate laboratory that could test for a specific genetic disorder. Inadequate counseling and testing for genetic disorders is a current problem. Therefore, in Japan, a well-established system of prenatal testing for genetic disorders and further educational programs for general physicians is required.

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Disclosure

The authors have no conflict of interest to declare.

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CLINICAL REPORT

AMERICAN JOURNAL OF medical genetics PART A

Radiological Clues to the Early Diagnosis of Hypochondroplasia in the Neonatal Period: Report of Two Patients

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Hypochondroplasia (HCH) is the mildest phenotype among fibroblast growth factor receptor 3 (*FGFR3*)-associated skeletal dysplasias. Affected individuals usually presents with mild short stature in preschool age. It was uncommon that a diagnosis of HCH is made in young affected children. Recently, however, prenatal ultrasound (US) has increased likelihood of detecting in utero mild short limbs. There have been a few reports on the early diagnosis of HCH in the neonatal period preceded by a suspicion of skeletal dysplasia on fetal US. However, the proper diagnosis of HCH is hampered by absence of the radiological criteria relevant to age, particularly those in the neonatal period. We report on the clinical and radiological findings in two HCH children with a *FGFR3* mutation. In both children, fetal US showed short femora and relatively increased biparietal diameter (BPD). However, postnatal assessment failed to make a specific diagnosis in the neonatal period. The correct diagnosis of HCH was accomplished by reassessment after exacerbation of postnatal short stature. In retrospective radiological review, the radiological findings relevant to HCH were discernible more easily in the neonatal period than at age of 3 years.

Key words: hypochondroplasia; radiological findings; neonatal period

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INTRODUCTION

Heterozygous, activating mutations of the fibroblast growth factor receptor 3 (*FGFR3*) are responsible for a group of skeletal dysplasias, such as achondroplasia (ACH), severe achondroplasia developmental delay and acanthosis nigricans (SADDAN), and thanatophoric dysplasia types I and II (TD I and II). Hypochondroplasia (HCH) is the mildest phenotype among *FGFR3*-associated skekeltal dysplasias. Affected individuals seek medical attention because of mild short stature and body disproportion usually in the preschool age. It was uncommon that the diagnosis of HCH is made in young affected children [Appan et al., 1990]. Recently, however, prenatal ultrasound (US) has increased likelihood of identifying in utero mild short limbs. The trend has brought a few reports on the early diagnosis of HCH in the neonatal period following prenatal suspicion of skeletal dysplasia.

The clinical diagnosis of HCH rests on identification of a few distinctive radiological findings, such as stubby long bones, lumbosacral interpediculate distance narrowing, short femoral necks, and an elongation of the fibula [Hall and Spranger, 1979; Matsui et al., 1998]. However, the solid diagnosis of HCH is hampered by absence of the radiological criteria relevant to age, particularly those in the neonatal period.

We report here on two HCH children with a *FGFR3* mutation, particularly focusing on US findings in the third trimester and postnatal radiological findings. Despite identification of short femora and relatively increased biparietal diameter (BPD) on prenatal US, postnatal assessment did not point to a diagnosis in the neonatal period, and the correct diagnosis was not made until 3 years of age. In retrospective radiological review, however, his

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radiological findings relevant to HCH were discernible more easily in the neonatal period than at age of 3 years.

PATIENTS AND METHODS

Patient 1: The boy was the first child born to healthy, nonconsanguineous Japanese parents with an unremarkable family history. Fetal US revealed nuchal translucency at 11 weeks of gestation. Chromosome banding of the amniotic fluid showed a normal male karyotype 46, XY. After 26 weeks of gestation, the femur length (FL) SD score showed a tendency to decline. The mother was referred at 32 weeks of gestation. Fetal US showed 1,630 g (−1.3 SD) of the estimated fetal weight, 8.2 cm (+0.4 SD) of BPD, 5.14 cm (−2.4 SD) of FL, and 4.59 cm (−3.4 SD) of humeral length (HL). There was no thoracic hypoplasia, calvarial abnormality, or deformity of the long bones. Then, BPD was within the normal range, but FL SD score was continuously decreased (Fig. 1a,b). He was born by spontaneous vaginal delivery at of 38 weeks and 3 days of gestation. Birth weight was 2,780 g (−0.2 SD), length 45.5 cm (−1.2 SD), head circumference 32.5 cm (−0.3 SD), and arm span-to-height ratio of 0.96. No external or internal malformations were evident. He was considered to be normal at that time. Follow-up was terminated at 5 months after birth because of normal development in early infancy.

By age 3 years, the patient's short stature was noted. His height was 85.4 cm (−3.1 SD), arm span was 83 cm, and upper segment length to lower segment length (U/L) ratio 1.67 (+4.5 SD). He had disproportionately short lower limbs and mildly bowed legs. His facial appearance was normal. Skeletal survey showed narrowing of the lumbar interpedicular distance (L1/L4: 1.07, normal: 0.89 ± 0.05, [Matsui et al., 1998]) and relative elongation of the fibula (fibula/tibia: 1.06, normal: 1.01 ± 0.02, [Matsui et al., 1998]) (Fig. 2a,b). The iliac wings are somewhat squared, the greater sciatic notches are mildly narrowed, and the femoral necks were also short (Fig. 2c). HCH was suspected based on the clinical and radiological findings. An analysis for *FGFR3* revealed a novel heterozygous L324V mutation (Fig. 3). The mutation was not found in his healthy parents or in 200 alleles derived from 100 normal controls. The L324 position is well preserved among FGFR families. Thus, L324V was considered to be a disease-causing mutation. In retrospective review of radiographs obtained in the neonatal period, we found squared ilia with short greater sciatic notches and horizontal

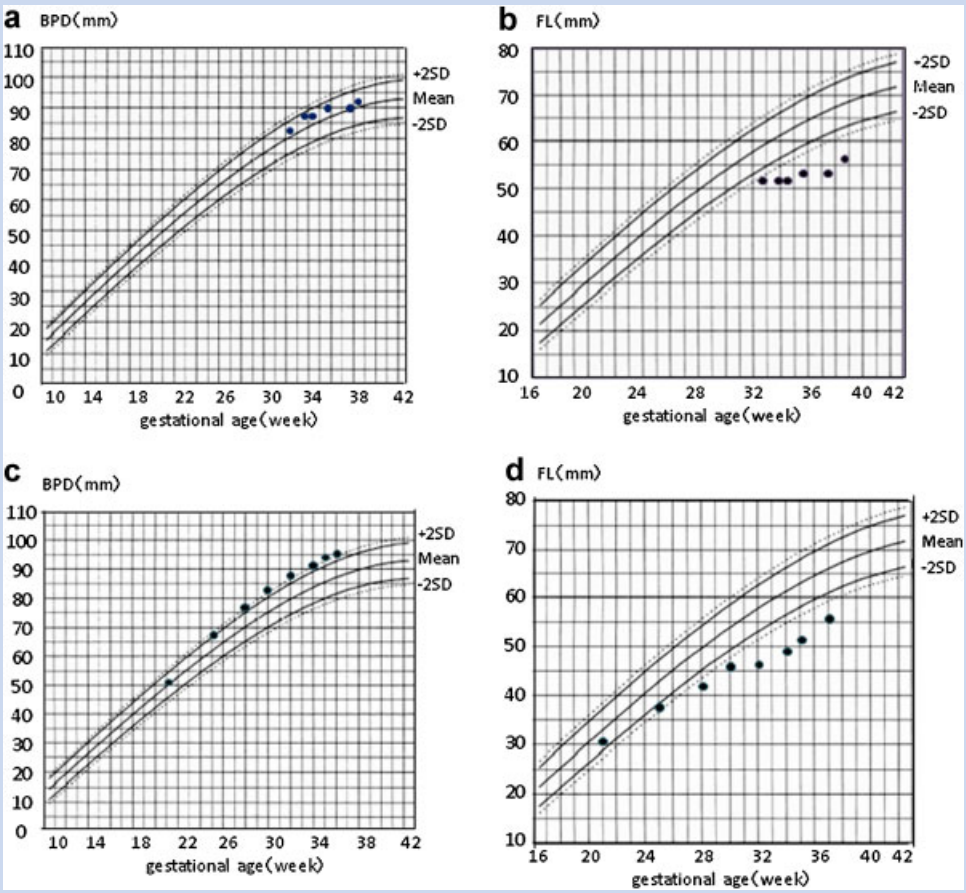

FIG. 1. Growth curve according to gestational age. a: Biparietal diameter (Patient 1), (b) femur length (Patient 1), (c) biparietal diameter (Patient 2), (d) femur length (Patient 2).

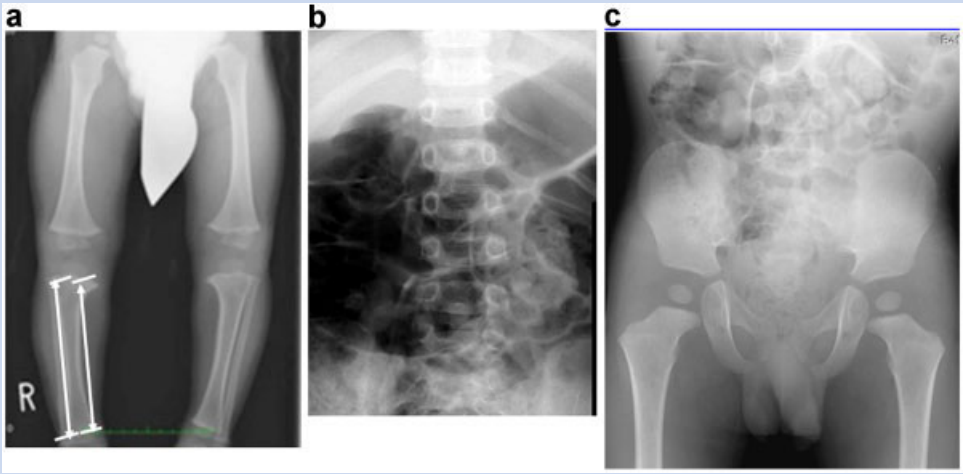

FIG. 2. The skeletal survey at 3 years of age in Patient 1. a: Relative elongation of the fibula, (b) narrowing of the lumbar interpedicular distance, c: slightly squared iliac wings and the mild shortening of greater sciatic notches.

acetabular roofs, and radiolucency of the femoral neck (Fig. 4b). However, lumbosacral interpedicular distance narrowing was not evident.

Patient 2: The boy was the second child born to healthy, non-consanguineous Japanese parents with an unremarkable family history. Decelerated growth of FL came to attention from 25 weeks of gestation. The mother was referred at 36 weeks of gestation. Fetal US revealed 2,164 g (−1.2 SD) of estimated fetal weight, 9.3 cm (1.3 SD) of BPD, and 5.78 cm (−2.7 SD) of FL. Then, femoral shortening was persistent, while BPD exceeded +2 SD of the average (Fig. 1c,d). He was born by spontaneous vaginal delivery at 40 weeks of gestation. Birth weight was 3,270 g (0.2 SD) and length was 49.0 cm (−0.4 SD). He was considered to be normal at that time.

At 3 years of age, he presented with short stature. Height was 85.7 cm (−3.0 SD), arm span 81 cm, and U/L ratio was 1.8 (+6.4 SD). He had disproportionately short limbs, but the facial appearance was normal. Skeletal survey showed narrowing of the lumbar interpedicular distance (L1/L4:1.02, normal: 0.89 ± 0.05, [Matsui et al., 1998]), relative elongation of the fibula (fibula/tibia: 1.07, normal: 1.01 ± 0.02, [Matsui et al., 1998]), and cupping of the distal femoral metaphysis. An analysis of the *FGFR3* gene showed a heterozygous N540K mutation, a common mutation in HCH. Retrospective review for radiographs in the neonatal period showed square ilia, shortening of the greater sciatic notches, horizontal acetabular roofs, and ovoid radiolucency of the femoral neck (Fig. 4c), but lumbar interpedicular distance narrowing was not overt.

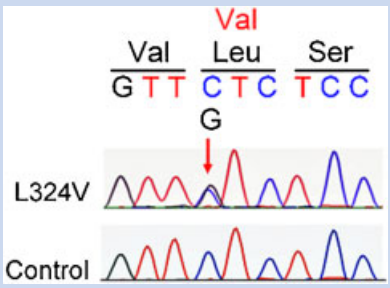

FIG. 3. *FGFR3* gene analysis of Patient 1. An electrochromatogram showing a heterozygous mutation (p.L324V) denoted by the arrows.

DISCUSSION

As exemplified by the present children, HCH tends to be overlooked in the neonatal period, because short stature and body disproportion in the disorder are not overt at that time [Appan et al., 1990]. Recently, there have been several reports on the early diagnosis of HCH, which was prompted by in utero identification of short femora on prenatal US [Jones et al., 1990; Huggins et al., 1999a; Kataoka et al., 2004; Bonnefoy et al., 2006; Karadimas et al., 2006]. Meticulous radiological interpretation is required to make a firm diagnosis of HCH in the neonatal period. The radiological findings of HCH are reviewed in many papers and standard textbooks [Jones, 2006]. However, it was problematic that the diagnostic criteria came from the observation of affected children in the preschool age.

In retrospective review of radiographs in the present children, we found that the pathognomonic findings for HCH were readily discernible in the neonatal period: The ilia were short and squared, the greater sciatic notches were short, and the femoral necks showed an oval radiolucency. These findings are essentially the same as, but much milder than, those of achondroplasia. The alterations of the ilia and proximal femora were more easily identifiable in the neonatal period than at 3 years of age. Lumbosacral interpediculate distance narrowing, the other important finding in HCH, was

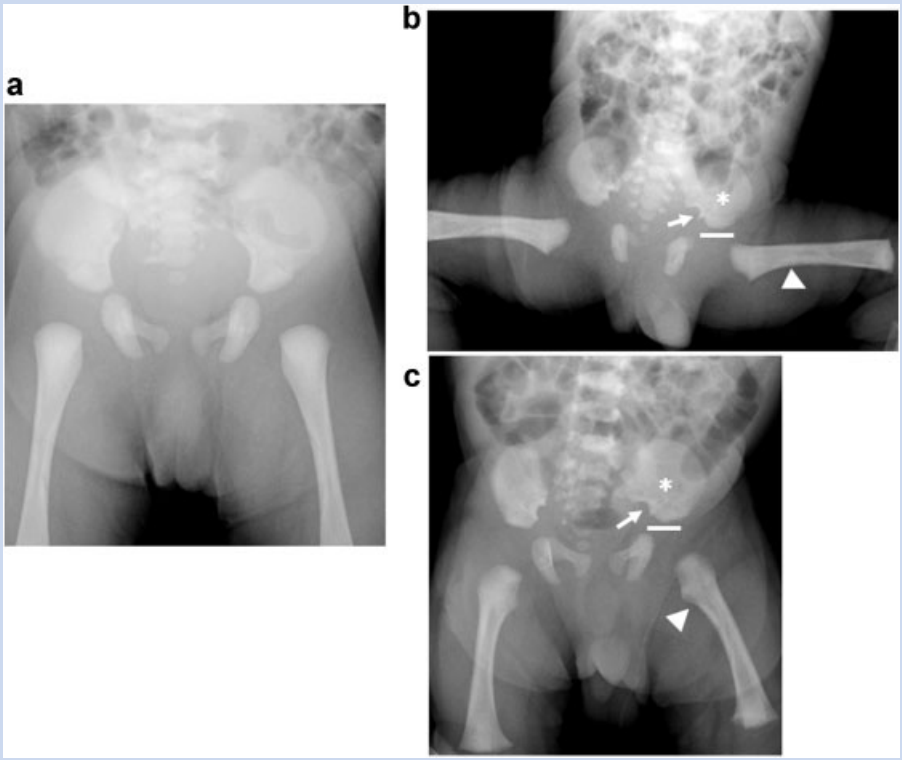

FIG. 4. A comparison of radiographs at birth with a healthy neonate. a: Normal neonate, (b) patient 1, (c) patient 2. The pelvic radiological findings of patients with HCH, showing square ilium (asterisk mark), narrowing of the greater sciatic notch (white arrow), and horizontal acetabular roofs (horizontal line), oval or square radiolucency of the femoral neck (white arrow head) which are more distinct compared with healthy neonate.

marginal in the neonatal period, while it was more overt at 3 years of age. We were able to see the changes in the ilia and proximal femora but not lumbosacral interpediculate distance narrowing in the illustrations of postmortem radiograph in a few clinical reports [Stoll et al., 1985]. In a report on a large series of HCH, Prinster et al. [1998] emphasized that the skeletal changes of the lumbar spine and long bones are crucial for the diagnosis, while those of the pelvis and hand are less important. However, their proposal is not true in the diagnosis for HCH neonates.

The prenatal diagnosis of HCH has been reported in familial cases at risk and isolated cases. Previously reported US markers of HCH are nonspecific findings, including decelerated growth curve of FL and a normal growth curve of BPD in the third trimester [Lemyre et al., 1999] [Karadimas et al., 2006]. There is a report, based on meticulous US measurement that distinguishes HCH fetuses from normal and achondroplastic fetuses [Huggins et al., 1999b]. In the present children, FL fell in the range from −2 SD to −3 SD in the third trimester. BPD was in the normal upper range in one child, while it exceeded +2 SD of the average in the other. Fetuses with significant discrepancy between SD scores of FL and BPD require meticulous postnatal radiological assessment.

HCH is genetically heterogeneous, and a number of HCH cases are not associated with *FGFR3* mutations. However, approximately 60% of HCH individuals result from a mutation in the intracellular FGFR3-tyrosine kinase domain. A minority of HCH mutations are found in the transmembrane or extracellular domain [Heuertz et al., 2006]. Previously reported *FGFR3* mutations in HCH are summarized in Table I. In this report, Patient 1 had a novel *FGFR3* mutation (L324V) that resided in the extracellular domain of FGFR3, and Patient 2 had a one of common mutations of HCH (N540K) that reside in the intracellular tyrosine kinase domain. As with other FGFR3 associated skeletal dysplasias (ACH, TD I and II, and SADDAN), HCH mutations give rise to constitutive activation of FGFR3. It is believed that intracellular mutations cause ligand-independent autophosphorylation, while extracellular or intramembranous mutations ligand-dependent increment of phosphorylation. On in vitro experiments, the level of activation is quantified by that of FGFR3 phosphorylation [Horton et al., 2007]. However, the high level of phosphorylation does not necessarily correspond to the severity of disease phenotypes. A recent report proposed that the phenotypic severity rests not only on the phosphorylation level but also on intercellular localization of abnormal FGFR3 [Harada et al., 2007]. HCH- and ACH-associated FGFR3s are transported to the plasma membrane, while TD- and SADDAN-associated FGFR3 are mostly accumulated in the endoplasmic reticulum (ER). ER localization is thought to cause ER stress and ultimate apoptosis of chondrocytes. It is tempting to assume that the phenotypic consequences come from the net-effects of activation levels of FGFs/FGFR3 signaling and severity of chondrocyte apoptosis. HCH mutations lead to the mildest

TABLE I. The Summary of Previously Reported *FGFR3* Mutations in HCH Cases

Amino-acid substitution	Domain	Reference
S84L	Ig I	Heuertz et al., 2006
R200C	Ig II	Heuertz et al., 2006
N262H	Ig II-IgIII linker	Heuertz et al., 2006
G268C	Ig II-IgIII linker	Heuertz et al., 2006
Y278C	Ig IIIa	Heuertz et al., 2006
L324V	Ig IIIa	Present case
N328I	Ig IIIa	Winterpacht et al., 2000
V381E	TM	Heuertz et al., 2006
I538V	TK1	Grigelioniené et al., 1998
N540K (70% of HCH cases)	TK1	Bellus et al., 1995
N540T	TK1	Deutz-Terlouw et al., 1998
N540S	TK1	Mortier et al., 2000
K650Q, K650N	TK2	Bellus et al., 2000

HCH: Hypochondroplasia; Ig: Immunoglobulin-like domains; TM: Transmembrane domain; TK1,2: Tyrosine kinase domain.

pathogenesis; i.e., membrane-localization and low phosphorylation levels. Despite the different mutation patterns between the present children, their clinical phenotypes were identical. The fact indicates that these mutations create the same level of ligand-dependent or ligand-independent phosphorylation of FGFR3.

In conclusion, awareness of the pathognomonic skeletal changes in the ilia and proximal femora described here allows for the early diagnosis of HCH in the neonatal period. Fetuses with short FL and upper normal or increased BPD on prenatal US are at risk for HCH. Such fetuses require meticulous postnatal radiological evaluation.

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ORIGINAL ARTICLE

Parental serum alkaline phosphatase activity as an auxiliary tool for prenatal diagnosis of hypophosphatasia

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ABSTRACT

Objective The objective of this study is to clarify the usefulness of parental alkaline phosphatase (ALP) for prenatal diagnosis of hypophosphatasia (HPP).

Methods Maternal (m) and paternal (p) ALP values were measured in 77 cases from a multicenter cohort (fetal skeletal dysplasia forum in Japan) of cases with short limbs on ultrasonography during pregnancy. After birth, X-rays, cord blood ALP, and gene analysis were evaluated to achieve an exact diagnosis. The screening usefulness of ALP was examined retrospectively.

Results Seventeen cases were eventually diagnosed as HPP and 60 as not HPP; the overall mean m-ALP and p-ALP (standard deviation) values were 133.4 (53) versus 197 (69) IU/L and 149.6 (71.8) versus 231 (61.4) IU/L ($p < 0.001$). Receiver operating characteristic curve analysis showed that the optimal m-ALP and p-ALP cutoff values were 123 and 165 IU/L, respectively. Presence of at least one of the m-ALP or p-ALP values abnormally low had a sensitivity, specificity, and positive predictive values of 82% (14/17), 93%, and 78%, respectively, for the diagnosis of HPP.

Conclusion Parental ALP measurement might be an auxiliary tool to hone in the prenatal diagnosis of fetal HPP.

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INTRODUCTION

Hypophosphatasia (HPP) is a well-known metabolic bone disease caused by a defect involving impaired mineralization with thin limbs and long bone bowing. Short limbs characterize the condition especially in the Japanese population. The perinatal type is defined as a life-threatening fetal disease.[1–3] Recently, new enzyme-replacement therapy after birth has been reported as a possible and promising therapy. Thus, some babies could be saved by this therapy without major sequelae.[4]

After birth, exact diagnosis of HPP can be performed by radiographic examination, enzyme activities, and gene analysis,[5–7] and, recently in Japan, clinical diagnosis by imaging after birth, gene analysis, and successful enzyme-replacement therapy has been reported.[8] As for the prenatal differential diagnosis of fetal skeletal dysplasia, ultrasonographic imaging is used first as a screening tool.[9,10] However, a definitive imaging diagnosis of HPP has not yet been established. Wenkert *et al.*[11] reported 15 different prenatal diagnoses, including type II osteogenesis imperfecta (OI) and campomelic dysplasia among 17 cases of benign-type HPP confirmed after birth. Given the new medical interventions available after birth, the focus has been on a more precise prenatal diagnosis. A definitive prenatal

diagnosis of HPP can be attained with tools other than imaging, such as parental and fetal gene analysis or measurement of cord blood alkaline phosphatase (ALP) activity. However, both methods are invasive and not cost-effective, given the large number of fetal skeletal dysplasias.

We analyzed the usefulness of parental blood ALP[12,13] as an auxiliary tool for the differential diagnosis of HPP from the many other fetal skeletal dysplasias and causes of short-limb fetuses in order to narrow down the differential diagnosis prior to proceeding to the diagnostic step, such as cord blood analysis of ALP, gene examination, and preparation of neonatal enzyme-replacement therapy. ALP activity increases gradually during pregnancy particularly in the third trimester, because of the contribution of the placental isoenzyme.[13] Thus, we have also assessed the effect of the trimesters of pregnancy on the screening efficacy of ALP measurement.

MATERIALS AND METHODS

This was a retrospective analysis of all cases submitted to the Fetal Skeletal Dysplasia Forum in Japan from 2007 to 2016. Such forum consists of a panel of voluntary experts in prenatal diagnosis.

Final diagnoses were made by radiographic analysis and gene analysis after birth, when indicated, to attain the final diagnoses of thanatophoric dysplasia (TD), OI, HPP, campomelic dysplasia, spondyloepiphyseal dysplasia congenita, and others. Maternal skeletal diseases with clear phenotype were not included.

In all cases, maternal ALP activity and paternal ALP activity if possible were measured. Maternal ALP activity was measured through the prenatal period just after the initial preliminary diagnosis of fetal bone disease. After final diagnosis, the parental ALP values and their accuracy for the exact diagnosis of fetal HPP were evaluated. ALP activity assays were performed in a few different ways. The value of the Japanese standard method, such as that of the Japanese Society of Clinical Chemistry,[14] was used, and the adult normal range has been established as 110–350 IU/L in every institute.

In our population, three major skeletal diseases are found more frequently: HPP, TD, and OI. Thus, the possibility of differential diagnosis was evaluated particularly focusing on these three diseases. Furthermore, the impact of gestational age on the accuracy of prenatal diagnosis was analyzed.

Statistical analysis was performed using SPSS version 20 (IBM Inc., Armonk, NY, USA) and PRISM version 5 (MDF Inc., Tokyo, Japan). A p value below 0.05 was considered significant. Receiver operating characteristic curve analysis was used to establish optimal screening thresholds of ALP values in maternal and paternal blood samples. All patients who registered with the fetal skeletal dysplasia forum provided written informed consent to participate in this study. The protocol was approved by the Institutional Review Board of Nagara Medical Center with regard to human rights and privacy issues.

RESULTS

There were 77 cases in the cohort, including 17 of HPP (Table 1), 6 of achondrodysplasia, 18 of TD, 24 of OI, 2 of spondyloepiphyseal dysplasia congenita, 2 of campomelic dysplasia, 3 of fetal growth restriction, and 5 other skeletal dysplasias (Figure 1). The mean ± standard deviation gestational age at measurement of maternal ALP was not significantly different between the HPP and non-HPP groups (26.7 ± 5.7 vs 25.7 ± 6.8 weeks, respectively; $p > 0.05$). Overall,

Table 1 **Parental serum ALP value and background of HPP cases**

Case	GW measure	Maternal ALP	Paternal ALP	Umbilical ALP	Prognosis	Genetic analysis
1	17	81	54	4	Still birth	
2[15]	18	160	250	2	Still birth	Paternal 1559delT, UPD
3	19	122	155	8	Still birth	1559delT, parent hetero
4	21	79	127	3	Still birth	
5	21	80	No data	No data	Still birth	
6	24	76	227	6	ND 37 weeks, 2119 g	
7	26	165	No data	No data	Alive, benign type	
8	27	62	No data	No data	Alive	
9	28	117	No data	6	ID 4 months	1559delT
10	29	165	121	7	Still birth 32 weeks, 1794 g	
11[8]	29	110	No data	0	Alive, ERT	exon12c 1471G >A
12	31	170	No data	3	ND, 31 weeks, perinatal lethal	1559delT
13	31	121	No data	0	ID 4 months	1559delT, parent hetero
14	32	240	110	82	Alive, ERT	
15	33	183	134	33	Alive, ERT	1559delT
16	33	108	300	205	Alive, benign type	exon7, hetero
17	35	229	118	9	ND 39 weeks, 2710 g	

ND, neonatal death; ID, infant death; ERT, enzyme-replacement therapy; hetero, heterozygous mutant; ALP, alkaline phosphatase; HPP, hypophosphatasia; GW, gestational weeks; UPD, uniparental disomy.

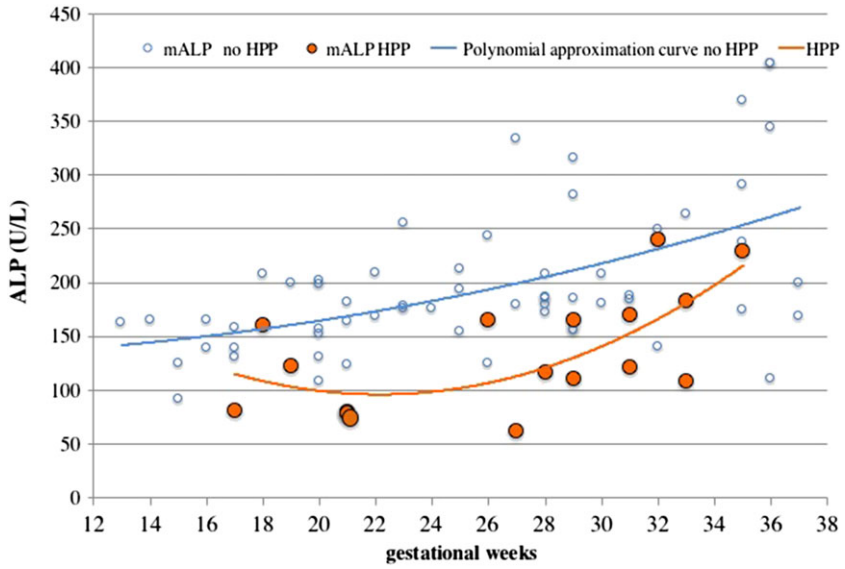

Figure 1 **All maternal alkaline phosphatase (mALP) values for short-limb cases including fetal hypophosphatasia (HPP) and other skeletal disease measured during pregnancy**

maternal ALP values were significantly lower in HPP than in non-HPP cases (133.4 ± 53 vs 197.4 ± 69 IU/L, $p < 0.001$). Significant differences were seen before 28 weeks of gestation (103.5 ± 40 vs 172.8 ± 46.6 IU/L, $p < 0.001$) and after 28 weeks (160.3 ± 50 vs 227.3 ± 80.5 IU/L, $p = 0.0016$) (Table 2).

Paternal ALP values were measured in 37 cases, and ALP values were significantly lower in HPP ($n = 10$) than in non-HPP cases (149.6 ± 72 vs 231 ± 61 IU/L, $p = 0.0016$).

Focusing on the three major skeletal dysplasias (HPP, OI, and TD), maternal ALP values showed significant differences between HPP and OI ($p < 0.001$, multiple comparison, post-hoc Tukey test). Importantly, during the second trimester, significantly different ALP values were noted between both HPP and OI ($p < 0.001$) and HPP and TD ($p < 0.05$). As for paternal ALP values, significant differences were noted between HPP and OI ($p < 0.05$) and between HPP and TD ($p < 0.001$) (Tables 3 and 4).

The optimal maternal ALP cutoff value for diagnosis was established by receiver operating characteristic curve analysis as 123 IU/L (any time during pregnancy), and the optimal paternal ALP value as 165 IU/L (Figure 2). When using 'at least one low value' diagnostic criterion based on these maternal and paternal cutoff values, the sensitivities throughout the entire pregnancy period, before 28 weeks of gestation, and after 28 weeks of gestation until 37 weeks of gestation were 82.4%, 93.3%, and 77.8%. The corresponding specificities were 75%, 93.9%, and 75%, with positive predictive values of 80%, 96.2%, and 88.9%, respectively (Table 5). Although the numbers were small, the paternal low ALP activity had high

Table 2 **Parental ALP values for HPP and non-HPP groups**

	Maternal ALP HPP all period	Maternal ALP no HPP all period	Statistics
n	17	60	
Mean (SD)	133.4 (53)	197 (69)	0.0008
Range	62–240	91–404	
	Maternal ALP HPP <28 weeks	Maternal ALP no HPP <28 weeks	
n	8	33	
Mean (SD)	103.5 (40.2)	172.8 (46.6)	0.0009
Range	62–165	91–333	
	Maternal ALP HPP 28–37 weeks	Maternal ALP no HPP 28–37 weeks	
n	9	27	
Mean (SD)	160.3 (50.48)	227.3 (80.5)	0.0167
Range	108–240	110–404	
	Paternal ALP HPP	Paternal ALP no HPP	
n	10	27	
Mean (SD)	149.6 (71.8)	231 (61.4)	0.0016
Range	54–300	130–429	

ALP, alkaline phosphatase; HPP, hypophosphatasia; SD, standard deviation.

Table 3 **Serum ALP value among three major diseases such as HPP, OI, and TD**

	n	Mean	SD	Median	Range
OI all period	24	216	91	192.5	91–404
TD all period	18	174	38.6	178.5	108–263
HPP all period	17	133.6	53	121	62–240
OI <28 weeks	14	179.5	62.8	161	91–333
TD <28 weeks	14	163.7	33.4	173	108–209
HPP <28 weeks	8	103.5	40.1	81	62–165
Paternal ALP OI	14	223.9	79.2	196	178–269
Paternal ALP TD	9	248.7	37.9	232	220–329
Paternal ALP HPP	10	179.6	72	127	54–300

HPP, hypophosphatasia; OI, osteogenesis imperfecta; TD, thanatophoric dysplasia; SD, standard deviation; ALP, alkaline phosphatase.

Table 4 **Multiple comparison (post-hoc Tukey test) of serum ALP value among three major diseases such as HPP, OI, and TD**

Factors (Tukey)	Mean difference	*q*	Significance	95% CI of difference
All period HPP vs TD	−40.3	2.46	ns	−96.12 to 15.52
All period HPP vs OI	−83.25	5.422	$p < 0.001$	−135.6 to −30.93
All period TD vs OI	−42.94	2.844	ns	−94.41 to 8.516
HPP <28 weeks vs TD <28 weeks	−60.21	3.975	$p < 0.05$	−112.8 to −7.609
HPP <28 weeks vs OI <28 weeks	−76	5.017	$p < 0.001$	−128.6 to −23.39
TD <28 weeks vs OI <28 weeks	−15.79	1.222	ns	−60.65 to 29.08
Paternal HPP vs paternal TD	−99.07	4.47	$p < 0.001$	−176.3 to −21.81
Paternal HPP vs paternal OI	−74.26	3.718	$p < 0.05$	−143.9 to −4.639
Paternal TD vs paternal OI	24.81	1.204	ns	−47.03 to 96.65

ALP, alkaline phosphatase; HPP, hypophosphatasia; OI, osteogenesis imperfecta; TD, thanatophoric dysplasia; CI, confidence interval; ns, not significant.

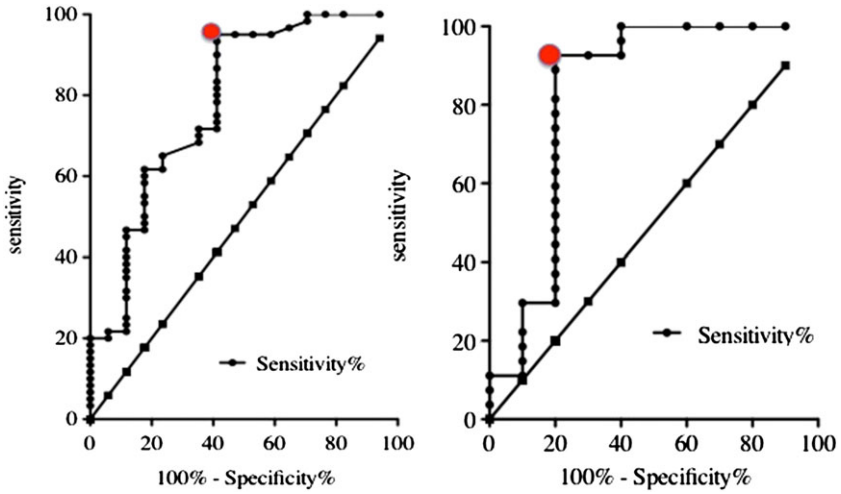

Figure 2 **Receiver operating characteristic curve of alkaline phosphatase (ALP) for the diagnosis of hypophosphatasia, with an optimal maternal ALP cutoff value of 123 and paternal ALP cutoff value of 165 [Colour figure can be viewed at wileyonlinelibrary.com]**

sensitivity, specificity, and positive predictive values of 80%, 92.6%, and 80%.

DISCUSSION

Recent medical developments such as enzyme-replacement therapy for HPP have increased survival even without sequelae.[4] It is thus important to reach a precise prenatal diagnosis of HPP. However, because of the many variations and limitations of fetal imaging, exact imaging criteria for the diagnosis for HPP have not yet been established. Although cord blood sampling for fetal ALP and parental gene analysis can allow accurate prenatal diagnosis, both methods are not easy to perform as first routine screening tests in cases of short fetal limbs because of their cost and invasiveness. We have found that parental blood ALP values provide a supportive tool to identify cases which may benefit from further diagnostic examination and preparation for enzyme-replacement therapy soon after delivery.

Because of the natural increase in maternal ALP values in late pregnancy both in normal and fetal HPP cases[13] due to ALP secretion from the placental isoenzyme, many investigators have abandoned using this method for prenatal diagnosis of the condition. However, the present results show the effectiveness of the 'at least one low' screening criterion (i.e. at least maternal and/or paternal ALP, cutoff below 123 and/or 165 IU/L, respectively). During the period of this study, 17 HPP cases were seen: paternal ALP activity was measured in 10 cases, and the positive rate of 'at least one low' was 82% (14/17 cases). Three cases showed both low maternal and paternal ALP values. Because paternal ALP is not affected by placental secretion of ALP, it can be measured at any time during the pregnancy. Indeed in our series, after 28 weeks, only 3/8 cases would have been detected by low maternal ALP measurement, whereas 4 cases would have been diagnosed using low paternal values. Thus, overall 7/8 cases would have been screened positive using 'at least one low' criterion.

An additional benefit of ALP is that the cost of performing this measurement is low as it is commonly used in daily clinical medicine. Only one case previously reported[15] showed normal ALP activity criteria for both parents. This case was found to have a paternal genomic abnormality of 1559 del and showed uniparental disomy.

Table 5 Diagnosis accuracy of fetal HPP of parental serum ALP activity

	HPP		No HPP		Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPA (%) (95% CI)	NPA (%) (95% CI)
	Low	Normal	Low	Normal				
At least 1 low in all period	14	3	4	56	82.4 (59–106)	93.3 (85–101)	77.8 (53–102)	94.9 (88–102)
Maternal low in all period	10	7	3	57	58.8 (29–89)	94.9 (88–102)	76.9 (48–106)	88.9 (79–98)
Paternal low in all period	8	2	2	25	80 (48–112)	92.6 (80–105)	80 (48–112)	92.6 (80–105)
At least 1 low <28 weeks	6	2	2	31	75 (37–113)	93.9 (84–104)	75 (37–113)	93.9 (84–104)
Maternal low <28 weeks	6	2	2	31	75 (37–113)	93.9 (84–104)	75 (37–113)	93.9 (84–104)
Paternal low <28 weeks	4	1	0	19	80 (35–125)	100 (1–1)	100 (1–1)	95 (83–107)
At least 1 low 28–37 weeks	8	2	1	25	80 (48–112)	96.2 (87–106)	88.9 (63–115)	92.6 (80–105)
Maternal low 28–37 weeks	14	3	5	26	82.4 (59–106)	83.9 (67–100)	73.7 (48–99)	89.7 (76–104)
Paternal low 28–37 weeks	8	1	2	25	88.9 (63–115)	92.6 (80–105)	80 (48–112)	96.2 (87–106)

At least 1 low, lower ALP either mother (ALP < 123) or father (ALP <165) using ROC cutoff, respectively.
PPA, positive predict accuracy; NPA, negative predict accuracy; ROC, receiver operating characteristic; ALP, alkaline phosphatase; HPP, hypophosphatasia; CI, confidence interval.

In considering the genetics of HPP, the mother, the father, or both can be carriers, as the condition follows either an autosomal recessive or – less commonly – an autosomal dominant inheritance. The carrier frequency is estimated to be 1/480 in Japan, based on genetic analyses.[16] Among all of the skeletal diseases, the Japanese population seems to have a higher incidence of fetal HPP. Mulivor *et al.*[1] noted that carriers apparently heterozygous for HPP had enzyme activity levels substantially lower than controls. A heterozygous carrier for HPP might show 50% ALP enzyme activity. So measurement of ALP activities in both parents would be useful for screening the carrier state and the HPP fetus.

Recently, a benign perinatal type of HPP has been diagnosed. Fetal diagnosis by imaging is also difficult for such mild phenotype cases.[12] Thus, ALP as a test is helpful after diagnosis of fetal skeletal dysplasia or bone disease by prenatal screening imaging. We successfully detected a case of benign type by measuring a maternal ALP value of 108 IU/L at 33 weeks of gestation (Table 2, case 16) although the fetus showed only slight bowing of the femur. Our method may contribute to detect benign perinatal types of HPP.

In light of our findings, a practical and cost-effective screening flow chart for HPP would include: (1) short-limb and thin head bone (clear brain imaging) on prenatal imaging and (2) ALP measurements of the parents with a low value of at least one (maternal and/or paternal). Cases fulfilling such criteria would benefit from more invasive or expensive prenatal diagnostic tests.

A limitation of this analysis is that a nomogram of ALP activity during the pregnant period has not been established in all countries outside of Japan. Additional studies are needed to confirm the value of parental ALP in the screening of HPP outside of Japan.

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WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- Prenatal diagnosis of fetal HPP is difficult to achieve.
- An accurate prenatal diagnosis is desirable because enzyme-replacement therapy after birth is beneficial.

WHAT DOES THIS STUDY ADD?

- In addition to fetal imaging, measuring parental ALP activity is useful to distinguish HPP from osteogenesis imperfecta and other fetal short-limb conditions throughout the whole period of pregnancy.
- Documentation of at least one parent with a low level of ALP seems to be a useful criterion to hone in the diagnosis of fetal HPP.
- ALP assay is not invasive, is inexpensive, and is easy to use.

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Occult fetomaternal hemorrhage in women with pathological placenta with respect to permeability

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Abstract

Aim: Women with pre-eclampsia (PE), placenta previa (PP), placental abruption (PA), and placental mesenchymal dysplasia (PMD) have been described as having placental permeability dysfunction. This study was performed to determine whether occult fetomaternal hemorrhage (FMH) is common in women with such complications and in women with non-reassuring fetal status.

Methods: Forty-one antenatal and 39 postnatal blood samples were obtained from 46 women, including 11 with placental permeability dysfunction (5, 3, 2, and 1 with PE, PP, PA, and PMD, respectively) and 35 controls without such complications. To estimate the amount of fetal red blood cells, flow cytometry was performed using the fetal cell count system with two antibodies against fetal hemoglobin and carbonic anhydrase and the β-γ system with two monoclonal antibodies against hemoglobin β-chain and hemoglobin γ-chain. A diagnosis of FMH was made when the fraction size of the isolated cell population on scatter plots expressing fetal hemoglobin alone or hemoglobin γ-chain alone accounted for ≥0.02% of the total cell population on scatter plots.

Results: FMH was identified in five women, including one each with PE, PA, PP, PMD, and no complications. Thus, the prevalence rate of FMH was significantly higher in women with complications than in controls (36% [4/11] vs 2.9% [1/35], respectively, $P = 0.009$). The FMH occurrence rate did not differ between women with and without non-reassuring fetal status (7.7% [1/13] vs 12% [4/33], respectively, $P = 1.000$).

Conclusion: The risk of fetal red blood cells trafficking into the maternal circulation may be increased in women complicated with PE, PA, PP, and PMD.

Key words: alpha-fetoprotein, fetomaternal hemorrhage, placenta previa, placental mesenchymal dysplasia, pre-eclampsia.

Introduction

Clinical fetomaternal hemorrhage (FMH) is rare, with an occurrence rate of 1 in 3000–10 000 women[1–4] and is suspected in women exhibiting clinical symptoms due to fetal anemia, such as non-reassuring fetal status (NRFS) on fetal heart rate (FHR) tracing, decreased fetal movement, and/or fetal hydrops. FMH of ≥ 30 mL and ≥ 80 mL occur in 1 in 333 women[5,6] and 1 in 1146 women,[1] respectively, and the majority of FMH cases of ≥ 30 mL occur with minimal clinical signs and symptoms.[1] Thus, occult FMH defined as FMH without any clinical signs may be relatively common among the general pregnant population.

The placenta is the interface between fetal and maternal circulations. Pre-eclampsia (PE), placenta previa (PP), placental abruption (PA), and placental mesenchymal dysplasia (PMD) are all conditions associated with placental pathology with respect to dysregulated interface function. Cell-free fetal DNA level is elevated in

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the maternal circulation in PE.[7] Cell-free human placental lactogen mRNA, which is produced entirely bytrophoblastic cells, is increased in the maternal circulation in PP.[8] The level of α-fetoprotein (AFP), the majority of which is produced by the fetal liver, is elevated in the maternal circulation before the occurrence of PA.[9] Aneurysmally dilated vessels on the fetal surfaces are characteristic features of PMD placentas,[10,11] in which AFP is markedly elevated in the maternal circulation.[10,12,13] These complications may allow more fetal red blood cell (RBC) trafficking into the maternal circulation compared to uncomplicated pregnancies. However, this issue has not been studied extensively.

The present study was conducted to test our hypothesis that occult FMH is relatively common in women complicated with PE, PP, PA, and PMD using flow cytometry, which is more sensitive for detection of fetal RBC than high-performance liquid chromatography or the acid elution method (Kleihauer–Betke test).[14] In addition, we examined whether occult FMH is common in cases with NRFS based on FHR tracing.

Methods

This study was approved by the Institutional Review Board of Hokkaido University Hospital and written informed consent was obtained from all pregnant participants prior to enrollment.

Participants

During the study period between June 2014 and February 2015, a total of 46 women participated in this study after giving informed consent. All of the women gave birth to singletons at our institution. Forty-one antenatal blood samples were obtained from 41 women within 25 days prior to delivery and 39 postnatal blood samples were obtained from 39 women within 3 days after delivery (Table 1). The demographic characteristics of the participants were obtained from medical records. Women were divided into two groups, with and without complications, including PE, PP, PA, and PMD. During the study period, there were 20, five, three, and one maternities at our institute complicated with PE alone, PP, PA, and PMD, respectively. These four complications were designated as placental diseases in this study. Sample size was determined by power analysis using Sample-Power 1.0 based on the following assumption: >30% of participants with placental diseases exhibit occult FMH, while <5% of women without placental diseases exhibit occult FMH.

Table 1 Demographic characteristics of 46 participants

	Placental diseases		
	Present (n = 11)	Absent (n = 35)	P-value
Age (years)	36.5 ± 4.3	33.9 ± 5.3	0.171
Antepartum NRFS	2 (18%)	4 (11%)	0.619
Intrapartum NRFS	0 (0%)	7 (20%)	0.171
Nulliparous	7 (64%)	22 (63%)	1.000
Placental diseases			
Pre-eclampsia†	5 (55%)	NA	
Placenta previa	3 (27%)	NA	
Placental abruption	2 (18%)	NA	
PMD	1 (9%)	NA	
Gestational weeks at delivery	32.4 ± 4.0	38.3 ± 1.7	0.165
<37	8 (73%)	1 (3%)	<0.001
<34	7 (64%)	1 (3%)	<0.001
Cesarean delivery	11 (100%)	20 (57%)	0.009
Birthweight (g)	1911 ± 923	2916 ± 571	0.002
1-min Apgar score < 8	8 (73%)	6 (17%)	0.001
5-min Apgar score < 8	0 (0%)	6 (17%)	0.311
Hb in the UCB (g/dL)	12.5 ± 4.0	14.6 ± 2.0	0.097
Blood sampling			
Antepartum	9 (82%)	32 (91%)	0.580
Post-partum	8 (73%)	32 (91%)	0.333
Fetomaternal hemorrhage	4 (36%)	1 (2.9%)	0.009

†There were six women with pre-eclampsia, but one with later onset of placental abruption was assigned to the placental abruption group. Hb, hemoglobin concentration; NA, not available; NRFS, non-reassuring fetal status based on fetal heart rate tracing; PMD, placental mesenchymal dysplasia; UCB, umbilical cord blood.

Preparation of blood samples for the detection of fetal RBC

The RBC collected from participants were washed three times in phosphate-buffered saline for 30 min in a solution containing formaldehyde, washed once more, and then permeabilized with sodium dodecyl sulfate for 3 min at room temperature. The flow cytometric study was then performed for detection of fetal RBC by two methods: (i) the fetal cell count (FCC) test using an FCC Kit (IQ Products BV) containing fluorescein isothiocyanate-labeled monoclonal mouse anti-human fetal hemoglobin (HbF) antibody and phycoerythrin-labeled polyclonal rabbit anti-human carbonic anhydrase antibody; and (ii) the β-γ test developed in our laboratory containing peridinin-chlorophyll protein complex – cyanine 5.5-conjugated monoclonal anti-human hemoglobin β antibody (hemoglobin β [37–8]: PerCP-Cy5.5; Santa Cruz Biotechnology) and fluorescein-conjugated monoclonal anti-human hemoglobin γ antibody (hemoglobin γ [51–7]: Santa Cruz Biotechnology).

Definition of FMH in this study

Molecules originating from fetal RBC were defined as an isolated cell population on scatter plots expressing HbF alone in the FCC system and/or an isolated cell population on scatter plots expressing hemoglobin γ-chain (Hb-γ) alone in the β-γ system. In our preliminary

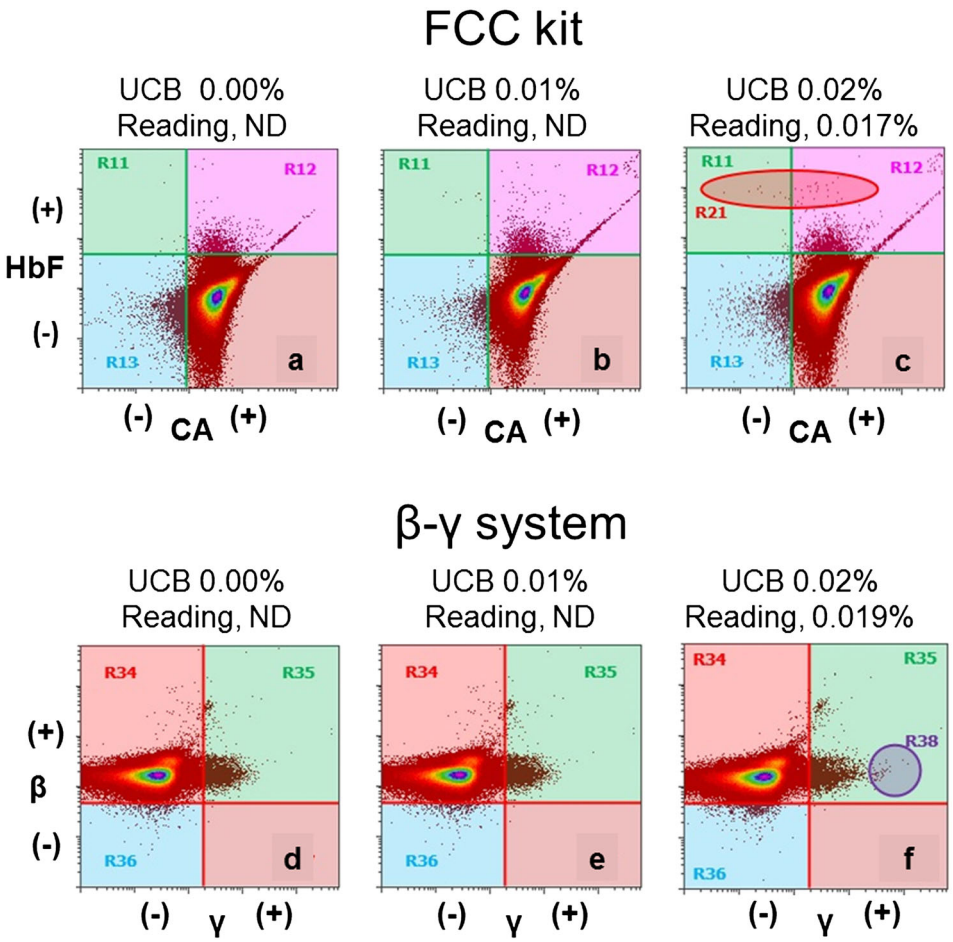

Figure 1 Flow cytometric study of male adult blood mixed with umbilical cord blood (UCB) in final UCB concentrations of 0.01% and 0.02% (vol/vol). Male adult blood containing 0.00%, 0.01%, and 0.02% UCB was used for examinations with the (a–c) fetal cell count (FCC) and (d–f) β-γ systems. No isolated scatter plot was identified in the blood containing 0.00% or 0.01% UCB in either the FCC or β-γ systems. However, the isolated scatter plot was identified in the blood containing 0.02% UCB, and the readings were 0.017% in the FCC and 0.019% in the β-γ system. CA, carbonic anhydrase; HbF, fetal hemoglobin. ND, not detectable isolated scatter plot.

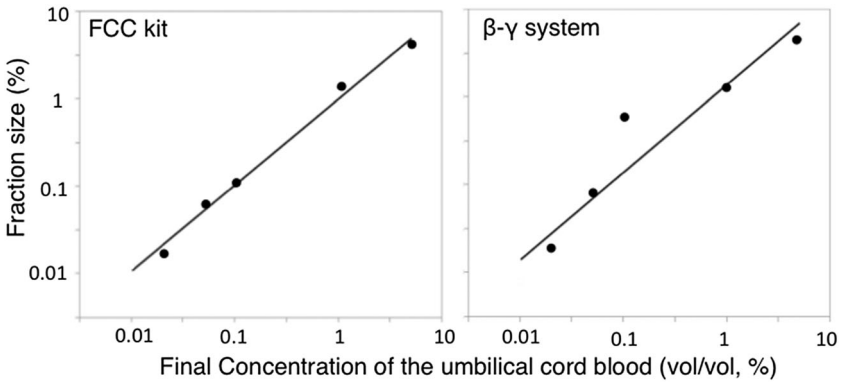

Figure 2 Association between actual measurements on flow cytometric systems and actual fractions of umbilical cord blood (UCB) in the mixed blood. The whole blood from a male adult volunteer was mixed with UCB obtained from a healthy term infant with parental consent to final UCB concentrations (vol/vol) of 0.01%, 0.02%, 0.05%, 0.1%, 1.0%, and 5.0%. The actual measurements on both the fetal cell count (FCC) and β-γ systems were well correlated with actual UCB concentrations of ≥ 0.02%. Thus, both systems were able to detect fetal red blood cells (RBC) in the whole blood containing ≥ 0.02% UCB.

experiments, an isolated cell population on scatter plots expressing HbF alone was identified in adult whole blood containing ≥ 0.02% (vol/vol) umbilical cord blood (UCB) in the FCC system (Fig. 1c). Similarly, the isolated cell population on scatter plots expressing Hb-γ alone was identified in adult whole blood containing ≥ 0.02% (vol/vol) UCB in the β-γ system (Fig. 1f). However, no isolated cell population on scatter plots was identified in adult whole blood containing 0.01% UCB in either system (Fig. 1b,e). In adult whole blood containing ≥ 0.02% UCB, actual readings of fraction sizes of isolated cell populations on scatter plots were correlated with UCB fraction size (Fig. 2). Therefore, a positive test result was defined as a fraction size of the isolated cell population on scatter plots expressing HbF alone ≥ 0.02% for the FCC system and that expressing Hb-γ alone ≥ 0.02% for the β-γ system. FMH was diagnosed in women with at least one positive test result with either antenatal or postnatal FCC or β-γ system.

Statistical analyses

Statistical analyses were performed using the JMP Pro 11 statistical software package (SAS). Differences in the means were tested using Wilcoxon's rank sum test between each group, and categorical variables were compared using Fisher's exact test. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

Results

Of the 46 women included in the study, six had PE (one with later PA was assigned to the PA group), three had PP, two experienced PA (one also had PE), and one had PMD. Thus, 11 of the 46 women had placental diseases (Table 1), and 11 of the 29 women with placental diseases during the study period participated in this study.

All of the 46 women underwent FCC or β-γ test at least once ante- and/or post-partum. Antenatal blood was

Table 2 Results of flow cytometric study

	Placental diseases		
	Present (n = 11)	Absent (n = 35)	P-value
Antenatal blood			
Positive on FCC system	2/9 (22%)	1/32 (3%)	0.116
Positive on β-γ system	2/9 (22%)	1/23 (4%)	0.184
Postnatal blood			
Positive on FCC system	3/8 (38%)	1/31 (3%)	0.022
Positive on β-γ system	2/5 (40%)	0/23 (0%)	0.027
No. of women with FMH†	4/11 (36%)	1/35 (2.9%)	0.009

†FMH was diagnosed in women with at least one positive test result. All 46 women underwent FCC or β-γ test at least once ante- and/or post-partum. Antenatal blood was examined with either FCC or β-γ system in 82% (9/11) and 91% (32/35) of women with and without placental diseases, respectively. Postnatal blood was examined with either FCC or β-γ system in 73% (8/11) and 91% (32/35) of women with and without placental diseases, respectively. After excluding three women with a positive test result on antenatal test(s) (two and one with and without placental diseases, respectively), postnatal blood was examined with either FCC or β-γ system in 67% (6/9) and 94% (32/34) of women with and without placental diseases, respectively. FCC, fetal cell count; FMH, fetomaternal hemorrhage.

Table 3 Details of five patients detected to have FMH

Case	Age (years)	Placental disease	Delivery		AFP (ng/mL)		HbF fraction				UCB [Hb] (g/dL)
							FCC		β-γ		
			GW	Mode	Antep	Post-p	Antep	Post-p	Antep	Post-p	
1†	38	PE/PA	27	CS	348	86	0.01%	0.04%	0.01%	0.01%	13.2
2†	26	PMD	30	CS	10 786	4990	NA	0.04%	NA	0.06%	8.3
3	36	PP	36	CS	447	258	0.11%	0.00%	0.09%	NA	14.5
4	37	None	39	TV	141	84	0.02%	0.02%	0.03%	NA	14.3
5†‡	43	PE/HELLP	31	CS	23 000	NA	3.03%	1.74%	2.45%	2.71%	3.4

†Nulliparous woman. ‡Non-reassuring fetal status on fetal heart rate tracing. AFP, α-fetoprotein; Antep, antepartum data; CS, cesarean section; FCC, fetal cell count; FMH, fetomaternal hemorrhage; GW, gestational week at delivery; Hb, hemoglobin; HbF, fetal hemoglobin; NA, not available blood samples; PA, placental abruption; PE, pre-eclampsia; PMD, placental mesenchymal dysplasia; PP, placenta previa; Post-p, post-partum data; TV, transvaginal delivery; UCB, umbilical cord blood.

examined with either FCC or β-γ system in 82% (9/11) and 91% (32/35) of women with and without placental diseases, respectively (Table 2). Among them, three women (two and one with and without placental diseases, respectively) exhibited positive test results on both tests (Cases 3, 4, and 5 in Table 3). Postnatal blood was examined with either the FCC or β-γ system in 73% (8/11) and 91% (32/35) of women with and without placental diseases, respectively. After excluding three women with a positive test result on antenatal tests, the postnatal tests covered 67% (6/9) and 94% (32/34) of women with and without placental diseases, respectively. An additional two women with placental diseases (Cases 1 and 2 in Table 3) exhibited a positive test result post-partum. Thus, four of the 11 women with placental diseases (36%) and one of the 35 without placental diseases (2.9%) exhibited at least one positive test result and were diagnosed with FMH ($P = 0.009$).

Actual scatter plot patterns and clinical details of the five FMH women are shown in Figure 3 and Table 3, respectively. Two cases (Cases 1 and 5) were complicated with PE, and each was complicated with PA (Case 1), PMD (Case 2), and PP (Case 3). Only Case 4 did not have placental diseases, but exhibited borderline positive test results three times. Clinical FMH occurred in two women (Cases 2 and 5) giving birth to anemic infants with UCB Hb concentrations of 8.3 and 3.4 g/dL, respectively.

Thirteen of the 46 women were judged as having NRFS according to the Japanese Guidelines for Obstetrical Practice[15] based on antenatal and or intrapartum FHR tracing (Table 1). FMH occurred in one (Case 5 in Table 3) of the 13 women with NRFS (7.7%), while it occurred in four of 33 women without NRFS (12%) ($P = 1.000$). Among the 35 women without placental diseases, 19 underwent cesarean section in the absence of labor pains and 16 experienced labor pains. FMH occurred in none of the former 19 women (0.0%) and one (Case 4 in Table 3) of the latter 16 women (6.3%) ($P = 0.457$).

Discussion

In this study, the prevalence rate of FMH was significantly higher for women with placental diseases than for those without placental diseases. After excluding three women with a positive test result on antenatal tests, among the remaining 43 women, postnatal tests did not cover 33% (3/9) and 5.9% (2/34) of women with and without placental diseases, respectively. However, this did not distort our conclusion; even on the assumption that these three and two women without postnatal tests

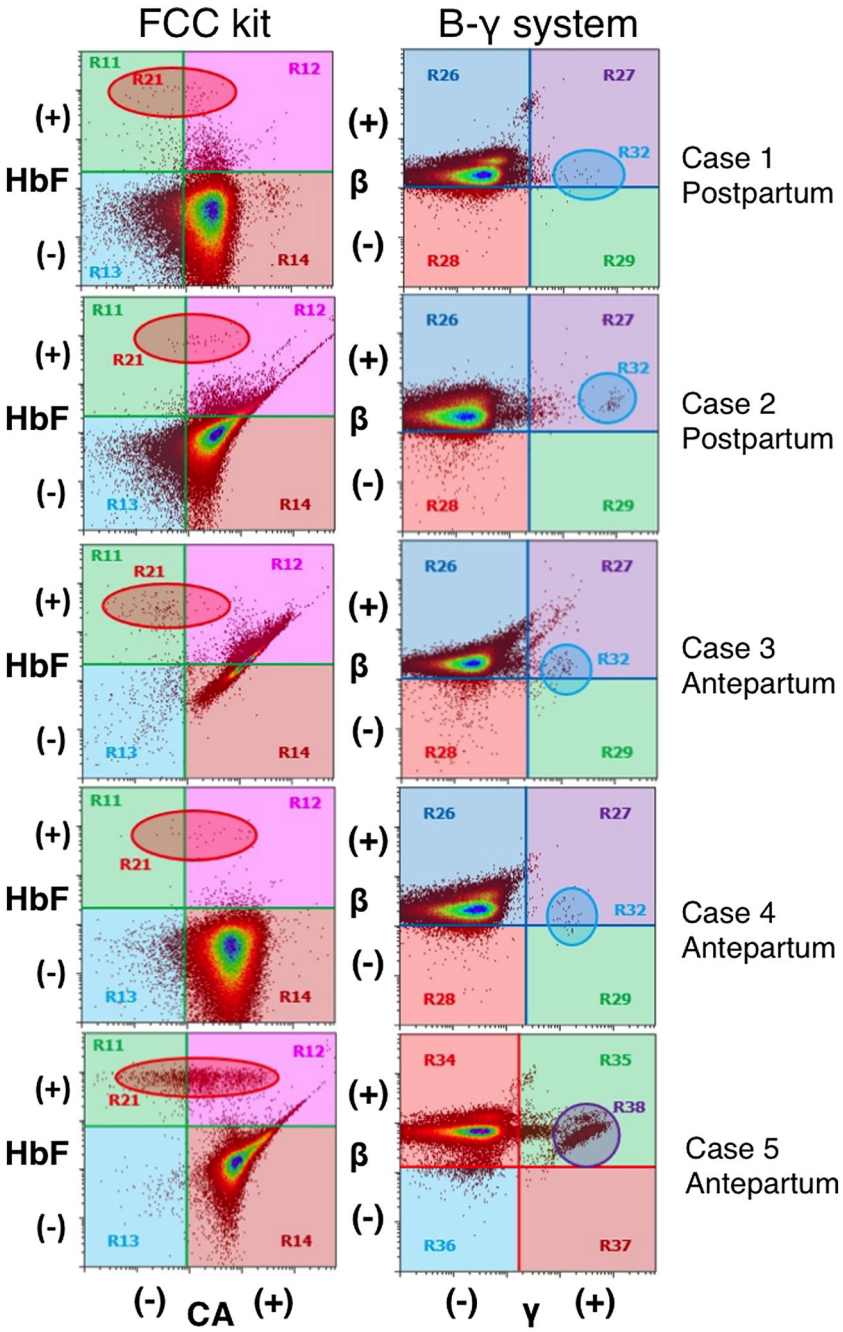

Figure 3 Five cases that were identified as having fetomaternal hemorrhage (FMH). The percentages of scatter plot populations with fetal hemoglobin expression alone and hemoglobin γ-chain expression alone were 0.04% and 0.01%, respectively, in the post-partum blood of Case 1. Corresponding values were 0.04% and 0.06%, respectively, in the post-partum blood of Case 2; 0.11% and 0.09%, respectively, in the antepartum blood of Case 3; 0.02% and 0.03%, respectively, in the antepartum blood of Case 4; and 3.03% and 2.45%, respectively, in the antepartum blood of Case 5 (this case was described previously in a case report[12]). CA, carbonic anhydrase; HbF, fetal hemoglobin.

exhibited exclusively negative and positive test results post-partum, respectively, the frequency of FMH would be significantly greater for women with than without placental diseases (36% [4/11] vs 8.6% [3/35], P = 0.0458). Thus, consistent with our hypothesis, these results suggested that perinatal fetal RBC trafficking into the maternal circulation was likely to occur in women with four complications: PE, PP, PA and PMD.

In this study, the prevalence rate of FMH was 2.9% (1/35) for control women without placental diseases, which was reasonable based on previous reports.[1,5,6,16] We set a cut-off point of 0.02% in this study for both the FCC and β-γ test systems. This implied that the FCC and β-γ systems gave a positive test result when ≥ approximately 1.0 mL of fetal blood was contained in 5000 mL of maternal circulating blood. The prevalence

rates of FMH of ≥ 150 mL, ≥ 80 mL, and ≥ 30 mL were estimated to be 0.2, 0.9, and 3 per 1000 births, respectively.[1,5,6] Thus, the incidence of FMH of ≥ 1.0 mL was expected to be far more prevalent, reaching several percent among the general population. Indeed, in a study examining the incidence of positive Kleihauer–Betke test results in low-risk, third-trimester, and asymptomatic women, as many as five of 98 (5.1%) had a positive test result, indicating FMH of 5 mL and 40 mL in four and one cases, respectively.[16] In another study examining the risk of FMH after external cephalic version at term for women with breech presentation using the FCC system,[17] although a higher cut-off point of 0.05% was used compared to our cut-off value of 0.02%, three occurrences of FMH were detected among 50 women (6.0%) after external cephalic version.[17] These three FMH women showed HbF fraction sizes of 0.06%, 0.08%, and 1.0%, respectively.[17]

In contrast to the FMH prevalence rate of 2.9% among control women, one of five women (20%) with PE alone, one of three with PP (33%), one of two with PA (50%), and one of one with PMD (100%) exhibited FMH. As described in the Methods section, we encountered 20, five, three, and one maternities complicated with PE alone, PP, PA and PMD, respectively, during the study period. Thus, 15 of 20 women with PE alone, two of five with PP, and one of three with PA did not participate in this study. However, even on the assumption that these background women with PE, PP, or PA participated in this study and exhibited exclusively negative test results on FCC and β-γ tests, the prevalence rate of FMH would be 5.0% (1/20) for women with PE, 20% (1/5) for those with PP, and 33% (1/3) for those with PA. Thus, it appeared that perinatal fetal RBC trafficking into the maternal circulation was likely to occur in women with these complications. In these four conditions, the placenta was suggested to have dysregulated function as an interface between fetal and maternal circulation as molecules derived from the fetus, such as cell-free fetal DNA, cell-free placental lactogen mRNA, and AFP, are often elevated in the maternal circulation in cases with these complications.[7–10,12,13] Thus, the present study confirmed dysregulated placental function with respect to permeability in women with such placental diseases.

However, relatively few studies have evaluated the correlations between these complications and the risk of FMH. In an earlier study by Christensen,[18] one of three women with relatively severe FMH was complicated with PE. In a study examining the risk of FMH in 14 women with PE and 11 control women using the FCC system,[19] the mean volume of FMH was significantly greater in women with PE than in controls (4.0 mL vs 1.0 mL, respectively).[17] Based on theoretical considerations, the prophylactic use of anti-D immune globulin is recommended in PP patients with antenatal hemorrhage.[20] PA is reported as a strong predictor of FMH.[2] In PMD pregnancies, as an extraordinarily higher risk of fetal death *in utero* is partially ascribed to FMH,[21] PMD was considered as an apparent risk factor for FMH.

In this study, the prevalence rate of FMH did not differ between women with and without NRFS. However, we expected a higher frequency of FMH in women with NRFS for several reasons, as follows. NRFS occurs frequently during parturition as a result of umbilical cord compression. Theoretically, the cord compression would cause fetal blood congestion within the placenta, as the return of fetal blood from the placenta via the umbilical vein is hampered compared to fetal blood flow into the placenta via the umbilical artery. The assumed fetal blood congestion within the placenta was expected to facilitate FMH. However, our results suggested that such a mechanism hardly caused fetal RBC trafficking into the maternal circulation. Indeed, none of seven intrapartum NRFS women showed positive FCC or β-γ test results in this study.

In conclusion, the present study suggested that FMH is common in patients with PE, PP, PA, and PMD. Most patients with FMH of ≤ 80 mL are asymptomatic[1] and the clinical outcomes may not be altered in these patients with minor FMH. However, it may be important to consider that women with PE, PP, PA, and PMD are prone to FMH.

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Disclosure

No author has any potential conflict of interest.

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Isolated gestational proteinuria preceding the diagnosis of preeclampsia – an observational study

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Conflict of interest
The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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Abstract

Introduction. Some pregnant women develop significant proteinuria in the absence of hypertension. However, clinical significance of isolated gestational proteinuria (IGP) is not well understood. This study aimed to determine the prevalence of IGP in singleton pregnancies and the proportion of women with IGP who subsequently developed preeclampsia (IGP-PE) among all PE cases. *Material and methods.* This was an observational study of 6819 women with singleton pregnancies at 12 centers, including 938 women with at least once determination of protein-to-creatinine ratio (P/Cr). Significant proteinuria in pregnancy (SPIP) was defined as P/Cr (mg/mg) level >0.27. IGP was defined as SPIP in the absence of hypertension. Gestational hypertension (GH) preceding preeclampsia (GH-PE) was defined as preeclampsia (PE) in which GH preceded SPIP. Simultaneous PE (S-PE) was defined as PE in which both SPIP and hypertension occurred simultaneously. *Results.* IGP and PE were diagnosed in 130 (1.9%) and 158 (2.3%) of 6819 women, respectively. Of 130 women with IGP, 32 (25%) progressed to PE and accounted for 20% of all women with PE. Hence, women with IGP had a relative risk of 13.1 (95% CI; 9.2–18.5) for developing PE compared with those without IGP [25% (32/130) vs. 1.9% (126/6689)]. At diagnosis of SPIP, P/Cr levels already exceeded 1.0 more often in women with S-PE than in those with IGP-PE [67% (33/49) vs. 44% (14/32), respectively, $p = 0.031$]. *Conclusions.* IGP is a risk factor for PE, and IGP-PE accounts for a considerable proportion (20%) of all PE.

Abbreviations: GH, gestational hypertension; GH-PE, gestational hypertension preceding preeclampsia; GW, gestational week; IGP, isolated gestational proteinuria; IGP-PE, IGP preceding PE; P/Cr, protein-to-creatinine ratio; PE, preeclampsia; S-PE, simultaneous PE; SPIP, significant proteinuria in pregnancy.

Introduction

Preeclampsia (PE) is a life-threatening complication (1) and occurs in approximately 2.3% of Japanese women (2). Hospitalized care and regular blood tests are currently recommended for women diagnosed with PE (3,4). A diagnosis of PE is made in women developing both hypertension and significant proteinuria in pregnancy (SPIP), although it has recently been proposed that PE can be diagnosed in hypertensive women with alterations in liver function, kidney function, and hematology in the absence of SPIP (5–7). The time between diagnosis of PE and delivery is relatively short, within 2 weeks in most cases (8,9). Early diagnosis of SPIP allows early diagnosis of PE and early initiation of intensive monitoring. Hospitalized care and regular blood tests would theoretically facilitate early detection of adverse conditions associated with PE, such as thrombocytopenia, liver and renal involvement, and posterior reversible encephalopathy syndrome, so leading to earlier delivery with improved outcomes, although evidence is lacking.

Maternal organ systems that are susceptible to excessive inflammation and endothelial damage in PE are the central nervous system, lungs, liver, kidneys, systemic vasculature, coagulation, and the heart (1). Therefore, clinical manifestations of PE vary between individuals; some exhibit SPIP first in the absence of hypertension, so-called "isolated gestational proteinuria" (IGP), develop hypertension later, and are then diagnosed with PE (8,9). Therefore, IGP is proposed to be an early clinical sign of PE (10). Hence, there are three types of PE: IGP preceding PE in which IGP precedes hypertension; gestational hypertension (GH) preceding PE, in which GH precedes SPIP; and PE in which hypertension and SPIP occur simultaneously (8,9,11). In a previous small single-center study (8), PE occurred in 19 of 37 women with IGP (51%) approximately 2 weeks after the confirmation of IGP (8), suggesting that IGP was a prominent risk factor for PE. However, there have been no studies assessing how many women develop IGP, how many women with IGP later develop hypertension, and what percentage of all PE cases are preceded by IGP. Addressing these issues may help to gain a better understanding of the nature of PE.

This multicenter retrospective observational study was conducted to determine the prevalence of IGP in singleton pregnancies and the proportion of women with IGP who subsequently developed PE (IGP-PE) among all PE cases.

Material and methods

This retrospective observational study was conducted after receiving approval from the Institutional Review Board of Hokkaido University Hospital (013-3999, 30 April 2014). The Japan Society of Obstetrics and Gynecology recommends 14–15 antenatal care visits before delivery and that pregnant women should undergo determination of blood pressure, proteinuria by dipstick test, and body weight at each visit (4). In this study, no definite protocols were predefined regarding the use of spot urine protein-to-creatinine ratio (P/Cr) test for confirmation of SPIP after obtaining dipstick test results. Therefore, the performance of the P/Cr test was at the attending physician's discretion. However, in 2014, the Japan Society of Obstetrics and Gynecology recommended use of the P/Cr test with a threshold of 0.27 mg/mg (corresponding to 30 mg/mmol) in women with the following dipstick test results: ≥1+ in the presence of hypertension, and ≥1+ on two successive antenatal care visits, and ≥2+ even in the absence of hypertension (4).

In this study, we abstracted the medical records of all 938 women with singleton pregnancies that underwent a P/Cr test at least once from a total of 6984 women (Figure 1). The database provided by the institutional central laboratory of participating facilities helped to identify women who had a P/Cr test at least once. SPIP was defined as P/Cr >0.27 mg/mg (corresponding to 30 mg/mmol) in spot urine specimen (3,4,6,7,12–14), and IGP was defined as SPIP in the absence of hypertension. Hypertension was defined as the occurrence of systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg and GH was defined as hypertension occurring on and after gestational week (GW) 20 in the absence of SPIP. PE was diagnosed in women who developed both hypertension and SPIP. Women with IGP or GH were followed up once or more per week on an outpatient basis, and were admitted to one of the 12 participating hospitals when they were diagnosed as having PE. The GW at new onset of hypertension and SPIP was specified in each subject, and PE was classified into one of three types according to the timing of the onsets of hypertension and SPIP: simultaneous PE (S-PE) in which both hypertension and SPIP were confirmed on the same day, IGP preceding PE (IGP-PE) in which IGP occurred

Key Message

Isolated gestational proteinuria, defined as protein: creatinine ratio >0.27 mg/mg (30 mg/mmol) in the absence of hypertension, is a prominent risk factor for developing preeclampsia (relative risk, 13.1 compared with women without isolated gestational proteinuria).

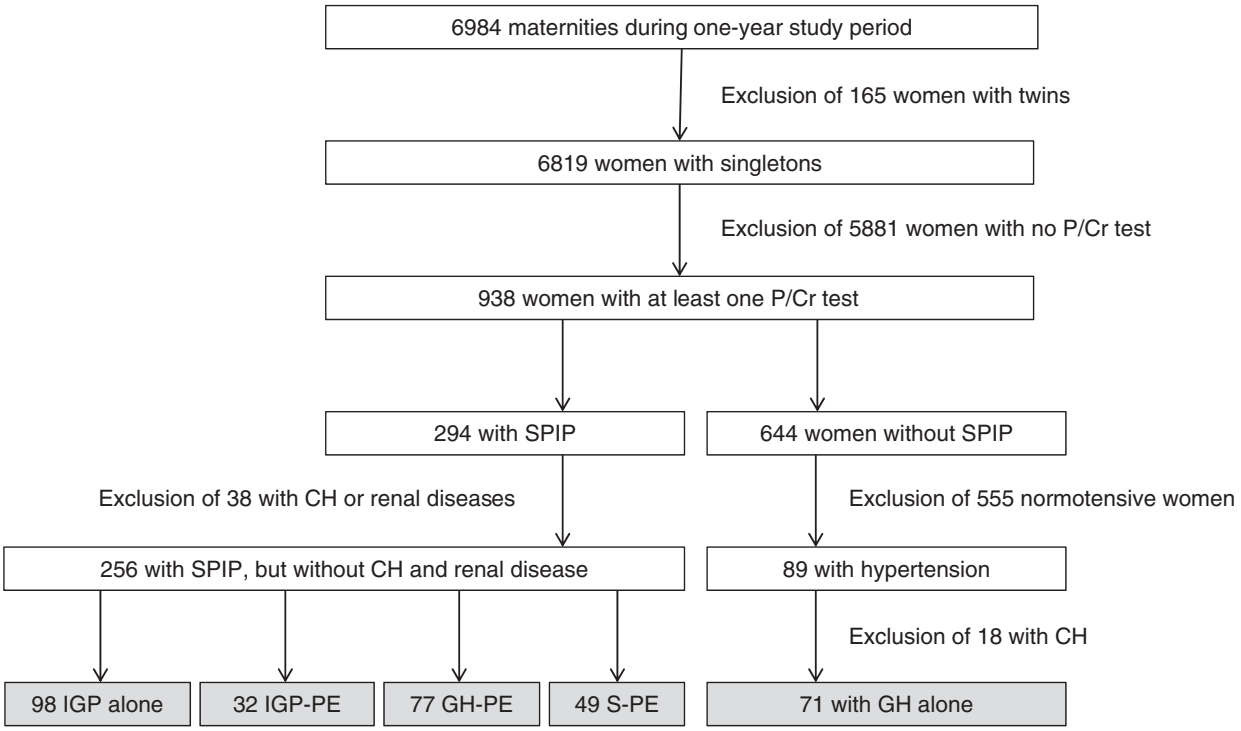

Figure 1. Flow diagram of the study participants. Shaded columns indicate 327 women who were selected from 6984 women after meeting all of the following four conditions: (1) singleton pregnancy, (2) new onset of SPIP or hypertension on and after GW 20, (3) no known renal diseases, and (4) no known hypertension. There were 6819 maternities carrying singletons at 12 centers during the 1-year study period between April 2014 and March 2015. The institutional central laboratory database identified 938 women with at least one P/Cr test, including 294 women with SPIP and 644 women without SPIP. The hospital discharge record database for the 644 women without SPIP identified 89 women with hypertensive disorders in pregnancy including chronic hypertension defined as hypertension confirmed before GW 20. The medical charts for each of the 294 women with SPIP and the 89 women with hypertension but without SPIP were reviewed. Gestational week at the new onset of SPIP and or hypertension was specified in the medical charts for each woman. Then, 56 women with known renal diseases, chronic hypertension, and or P/Cr >0.27 before GW 20 were excluded. GH, gestational hypertension; GH-PE, GH preceding preeclampsia (PE); GW, gestational week; IGP, isolated gestational proteinuria; IGP-PE, IGP preceding PE; P/Cr, protein-to-creatinine ratio; SPIP, significant proteinuria in pregnancy (defined as P/Cr >0.27); S-PE, simultaneous PE.

earlier than hypertension, and GH preceding PE (GH-PE) in which hypertension occurred earlier than SPIP.

In this study, no definite protocols were predefined regarding the use of induction of labor in women with PE. Therefore, women with PE were managed according to the policies at each institution. The Japan Society of Obstetrics and Gynecology recommends early delivery in women with PE who have one or more of the following conditions (4): uncontrollable hypertension exceeding systolic blood pressure ≥180 mmHg and/or diastolic blood pressure ≥ 110 mmHg even with antihypertensive agents; net weight gain ≥3.0 kg per week; liver dysfunction as evidenced by elevated aspartate aminotransferase (>45 IU/L) level and/or elevated level of lactate dehydrogenase (>400 IU/L) concomitant with reduced platelet count ($<100 \times 10^9$/L) and/or reduced antithrombin activity level (<60% of normal activity level); and an increase in proteinuria with P/Cr test result >5.0.

The following 12 facilities located throughout Japan participated in this study: Hiroshima University Hospital, Rakuwakai Otowa Hospital, Ohno Ladies Clinic, Mie Chuo Medical Center, Mie University Hospital, Nippon Medical School Tama Nagayama Hospital, Juntendo University Hospital, University of Tsukuba Hospital, Jichi Medical University Hospital, Fukushima Medical University Hospital, Hakodate Central General Hospital, and Hokkaido University Hospital. The clinical data, including patient age, parity, and clinical outcomes, were obtained from medical charts of women with IGP, GH, and PE.

Statistical analyses

Data are presented as median (range). Statistical analyses were performed using the JMP Pro11© statistical software package (SAS, Cary, NC). The Tukey–Kramer method

Table 1. Demographic characteristics of 327 women.

	IGP (n = 130)		GH (n = 148)		
Final diagnosis	IGP (n = 98)	IGP-PE (n = 32)	GH (n = 71)	GH-PE (n = 77)	S-PE (n = 49)
Maternal age (years)	31 (18–44)	33 (19–42)	34 (17–45)	35 (19–45)	34 (16–41)
Nulliparous women	61 (62.2%)	23 (71.9%)	40 (56.3%)	46 (59.7%)[a]	37 (75.5%)[a]
GW at onset					
SPIP	35.0 (21.6–40.9)	33.3 (24.7–39.6)	NA	35.0 (23.9–PPW 0.9)	36.7 (23.6–41.1)
Hypertension	NA	36.0 (24.9–40.4)	35.9 (20.0–40.3)	33.7 (23.4–40.4)[a]	36.7 (23.6–41.1)[a]
Interval (weeks)[f]	2.2 (−1.0 to 17.4)[a,b]	1.3 (0.2–11.7)[c,d]	NA	0.5 (0.0–7.0)[a,c]	0.3 (0.0–6.9)[b,d]
GW at delivery	38.8 (23.9–41.6)[a,b]	37.0 (25.3–40.7)	37.9 (27.0–41.6)[c]	36.4 (24.1–41.3)[a,c]	37.1 (23.7–41.3)[b]
<37	20 (20.4%)[a,b]	16 (50.0%)[c]	19 (26.8%)[c,d,e]	44 (57.1%)[a,d]	22 (44.9%)[b,e]
Birthweight (kg)	2.91 (0.62–3.98)[a,b,c]	2.20 (0.37–3.44)[a,d]	2.80 (0.67–4.01)[d,e]	2.15 (0.38–3.79)[b,e]	2.56 (0.44–4.06)[c]

Data are presented as the median (range) or number (percentage).
Significant difference was compared between three groups including IGP-PE, GH-PE and S-PE; $p < 0.05$ between two figures with superscript letters [a–e]. [f]Time interval until delivery after SPIP confirmation.
GH, gestational hypertension; IGP, isolated gestational proteinuria; GW, gestational week; GH-PE, hypertension-preceding preeclampsia; IGP-PE, proteinuria-preceding preeclampsia; PPW, postpartum week; S-PE, simultaneous preeclampsia; SPIP, significant proteinuria in pregnancy. The 130 women with IGP developed SPIP at GW 34.9 (21.6–40.9) and 148 women with GH developed hypertension at GW 34.4 (20.0–40.4). SPIP was confirmed postpartum for the first time in 15 of the 77 women with GH-PE.

was used for comparison of medians. Pearson's chi-squared test was used for comparison of categorical variables. In all analyses, $p < 0.05$ was taken to indicate statistical significance.

Results

The flow of study participants is shown in Figure 1. A total of 130 women were identified as having IGP, corresponding to 1.9% (130/6819) of the background pregnant population (n = 6819) (Figure 1). Thirty-two (25%) of the 130 women with IGP later developed hypertension and were diagnosed with IGP-PE. GH-PE and S-PE were diagnosed in 77 and 49 of the 327 women included in the study population, respectively. Hence, women with IGP-PE accounted for 20% (32/158) of all women with PE. The total number of PE cases, i.e. 158, corresponded to 2.3% (158/6819) of the background population. Compared with the 6689 women who were not diagnosed as having IGP, the risk of developing PE was significantly greater in the 130 women with IGP [25% (32/130) vs. 1.9% (126/6689), respectively, $p < 0.0001$] and the relative risk of developing PE was 13.1 (95% CI 9.2–18.5) for women with IGP.

The clinical characteristics were compared between groups (Table 1). Hypertension was diagnosed significantly earlier in the GH-PE group than the S-PE group. GW at the diagnosis of SPIP did not differ significantly between groups. The time interval between confirmation of SPIP and delivery was significantly longer in the IGP-PE group than in the other two PE groups. SPIP was confirmed postpartum for the first time in 15 of the 77 women (19%) with GH-PE. There were no significant differences in variables shown in Table 1 between 62 and 15 women with GH-PE with SPIP confirmed antepartum and postpartum, respectively, except for the GW at onset

Table 2. P/Cr tests performed in three groups.

	Final diagnosis		
	IGP-PE	GH-PE	S-PE
No. of women	32	77	49
Total no. of P/Cr tests	96	188	125
No. of P/Cr tests/person	3 (1–5)	2 (1–5)	2 (1–5)
Once	6 (18.8%)	18 (23.4%)	14 (28.6%)
Twice	4 (12.5%)	23 (29.9%)	12 (24.5%)
Three times or more	22 (68.8%)[a]	36 (46.8%)[a]	23 (46.9%)
When SPIP was diagnosed			
[P] (mg/dL)	98 (10–683)	74 (9.1–3890)	158 (9.0–2396)
[Cr] (mg/dL)	122 (26–335)	91 (18–401)	119 (20–374)
P/Cr (mg/mg)	0.81 (0.32–5.6)	0.92 (0.27–21.7)	1.48 (0.27–13.8)
<0.5	9 (28.1%)	24 (31.2%)[a]	7 (14.3%)[a]
>1.0	14 (43.8%)[a]	34 (44.2%)[b]	33 (67.4%)[a,b]
>1.5	10 (31.3%)	27 (35.1%)	24 (49.0%)
>2.0	6 (18.8%)	24 (31.2%)	19 (38.8%)
Dipstick test result			
Negative/ equivocal	1 (3.1%)	13 (16.9%)	3 (6.1%)
1+	10 (31.3%)	20 (26.0%)	9 (18.4%)
≥2+	21 (65.6%)	44 (57.1%)[a]	37 (75.5%)[a]

Data are presented as the median (range) or number (percentage).
[Cr], creatinine concentration; GH-PE, hypertension-preceding preeclampsia; IGP-PE, proteinuria-preceding preeclampsia; [P], protein concentration; P/Cr, protein-to-creatinine ratio; S-PE, simultaneous preeclampsia; SPIP, significant proteinuria in pregnancy.
$P < 0.05$ between two figures with superscript letters.

of hypertension (32.7 ± 4.0 vs. 35.1 ± 3.4, respectively, $p = 0.03$). Hence, women with GH-PE with antepartum SPIP developed hypertension earlier compared with those with postpartum SPIP.

In this study, SPIP was diagnosed in women with P/Cr > 0.27. The P/Cr test was performed 96, 188, and 125 times for 32, 77, and 49 women diagnosed with IGP-PE, GH-PE, and S-PE, respectively (Table 2). The number of women with ≥3 P/Cr tests was significantly greater in the IGP-PE group than in the other two PE groups. There were no significant differences in protein concentration, creatinine concentration, or P/Cr level at diagnosis of SPIP between the three PE groups. However, the number of women with a P/Cr level >1.0 at diagnosis of SPIP was significantly greater in the S-PE group than in the other two PE groups, although GW at the diagnosis of SPIP did not differ significantly between groups; 67% (33/49) vs. 44% (14/32) with $p = 0.03$ for S-PE vs. IGP-PE, respectively and 67% [33/49] vs. 44% [34/77] with $p = 0.009$ for S-PE vs. GH-PE, respectively (Table 2, Figure 2b).

The P/Cr level appeared to increase with advancing gestational age in most women with PE, although the rate of increase in P/Cr level (per unit time) varied greatly

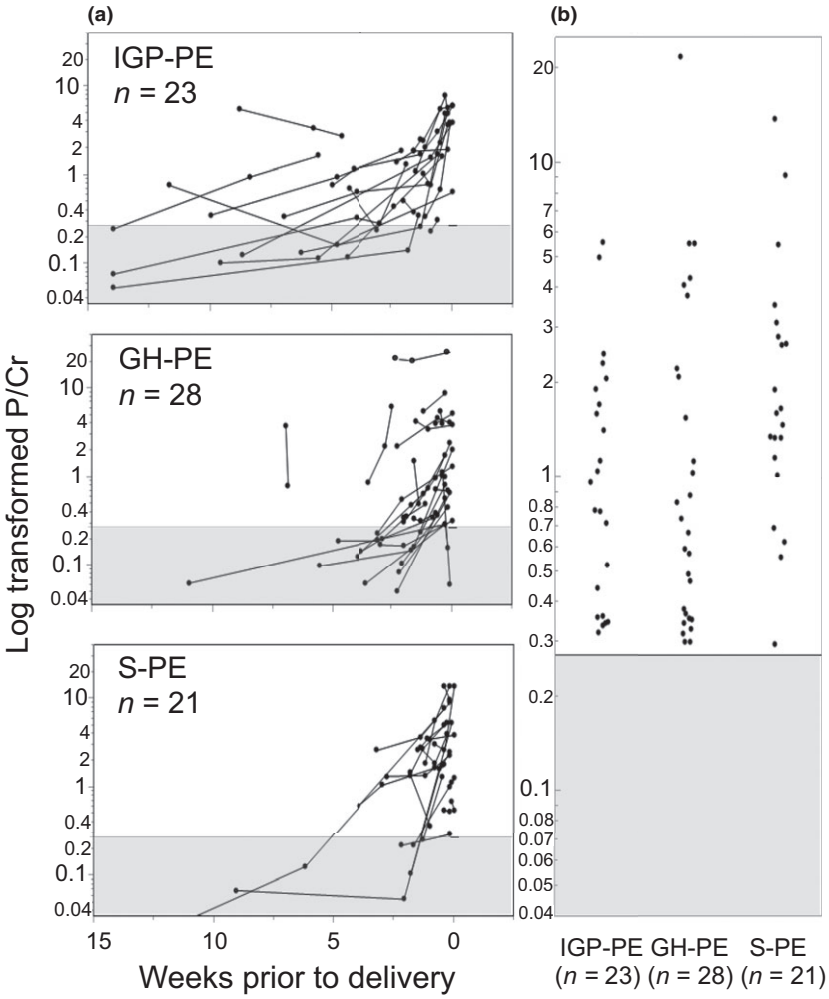

Figure 2. Antenatal changes in P/Cr ratio (a) and P/Cr ratio at the diagnosis of SPIP (b). (a) The shaded area indicates P/Cr ratio <0.27. Twenty-three of 32 women with IGP-PE, 28 of 77 women with GH-PE, and 21 of 49 women with S-PE underwent P/Cr test at least twice antepartum, and were shown to have SPIP antepartum (15 of the 77 women with GH-PE exhibited SPIP postpartum for the first time). The P/Cr ratio increased with advancing gestation in most women with PE, although the rate of increase of proteinuria varied greatly between individuals. (b) Distribution of P/Cr ratio at the diagnosis of SPIP is shown. Number of women with P/Cr >1.0 is indicated in rectangles. A significantly greater number of women exhibited P/Cr >1.0 at the diagnosis of SPIP in women with S-PE (67%; 33/49) than in those with IGP-PE (44%; 14/32) and GH-PE (44%; 34/77). GH, gestational hypertension; GH-PE, GH preceding preeclampsia (PE); IGP-PE, isolated gestational proteinuria preceding PE; P/Cr, protein-to-creatinine ratio; SPIP, significant proteinuria in pregnancy (defined as P/Cr >0.27); S-PE, simultaneous PE.

between individuals (Figure 2a). Distribution of P/Cr level at the diagnosis of SPIP is shown in Figure 2(b).

Discussion

In this study, 1.9% (130/6819) of women with singleton pregnancies exhibited IGP defined as P/Cr level >0.27 in the spot urine sample in the absence of hypertension, 25% (32/130) of women with IGP later progressed to PE, and IGP-PE accounted for 20% (32/158) of all women with PE. In a recent study by Sarno et al. (15), IGP-PE accounted for 25% of all 195 women with PE.

The reference standard for diagnosis of SPIP is proteinuria ≥300 mg in 24-h urine (5–7), although PE can be diagnosed without confirmation of SPIP in hypertensive women with certain clinical conditions according to recent guidelines (5–7). We speculated that problems inherent in 24-h urine collection may have prevented determination of the numbers of women exhibiting IGP, the numbers of women with IGP progressing to PE, and the percentage of IGP preceding PE in all cases of PE. To our knowledge, most guidelines (3,5–7) for the diagnosis and treatment of hypertensive disorders in pregnancy do not mention the prevalence of IGP because there is a lack of reports in the literature. This lack of reports may be a result of difficulty in performing studies addressing these issues for several reasons. Convenient P/Cr testing using random urine samples has only recently been introduced in obstetric practice as an alternative to 24-h urine collection (3–7). The rate of false-positive test results on dipstick tests is high because of the inherent nature of this primary screening tool for SPIP, especially in concentrated urine samples (16–19). Although 24-h urine collection has been mandatory for diagnosis of SPIP, it is frequently incomplete and inconvenient for pregnant women (18–20); therefore, physicians may be reluctant to ask women for repeated 24-h urine collection.

Our study design did not preclude the possibility that there may have been some women with IGP among the 5881 women that did not undergo P/Cr testing at all during pregnancy (Figure 1). However, it was notable that there were at least 130 women with IGP, corresponding to 1.9% of the whole background population. In a recent study by Ekiz et al. defining SPIP as protein loss ≥300 mg in 24-h urine samples (21), a much lower IGP prevalence rate of 0.5% (157/31 472) was reported. We speculate that this difference in IGP prevalence rate may have been a result of difficulty in performing the 24-h urine collection test. The P/Cr test was performed in 938 of all 6819 women (14%) (Figure 1) and 409 times in the 158 women with PE before and/or after the diagnosis of PE (Table 2) in this study.

The risk of PE among women with IGP was 25% (32/130) in this study, which was relatively consistent with that of 34% (53/157) in the study by Ekiz et al. (21). This clearly indicated that IGP is a risk factor for PE. The number of women diagnosed with PE in our setting corresponded to 2.3% of the whole background population of 6819 women, which was consistent with the results of a previous study in which the prevalence rate of PE based on traditional criteria for diagnosis was 2.3% among 301 735 pregnant Japanese women with singleton pregnancies (2). The risk of PE was estimated as 1.9% (128/6689) among women not diagnosed with IGP in this study, which was much lower than the rate of 25% among women with IGP, indicating a relative risk of 13.1 for developing PE in women with IGP. Thus, IGP was an apparent prominent risk factor for PE in this study.

In this study, the number of women with a P/Cr level >1.0 at diagnosis of SPIP was significantly greater in the S-PE group than in the other two PE groups (Table 2, Figure 2b). Three previous studies (8,9,22) suggested that proteinuria increased with advancing gestational age. However, it was suggested that the rate of increase in proteinuria varied greatly between individuals in this study (Figure 2a).

In conclusion, the present study demonstrated that IGP, defined as P/Cr >0.27 (30 mg/mmol) in the absence of hypertension, is a risk factor for developing PE. IGP-PE accounts for a considerable proportion (20%) of all cases of PE. This knowledge may contribute to earlier diagnosis of PE and earlier delivery when indicated to improve outcomes in such women with IGP-PE.

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Characteristic calcaneal ossification: an additional early radiographic finding in infants with fibrodysplasia ossificans progressiva

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ORIGINAL ARTICLE

Characteristic calcaneal ossification: an additional early radiographic finding in infants with fibrodysplasia ossificans progressiva

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Abstract
Background We have clinically encountered children with fibrodysplasia ossificans progressiva who had abnormal calcaneal ossification.
Objective To evaluate whether calcaneal ossification variants are significant radiographic findings in children with fibrodysplasia ossificans progressiva.
Materials and methods Lateral feet radiographs in nine children who fulfilled the diagnostic criteria of fibrodysplasia ossificans progressiva were reviewed. The studies were obtained during infancy or early childhood.
Results Fourteen lateral foot radiographs of fibrodysplasia ossificans progressiva were available for this study (ages at examination: 1-104 months). Four children ages 2 months to 11 months showed double calcaneal ossification centers; 7 children had plantar calcaneal spurs that decreased in size with age. Overall, eight of nine children with fibrodysplasia ossificans progressiva demonstrated double calcaneal ossifications and/or plantar calcaneal spurs in infancy or childhood.
Conclusion Double calcaneal ossification centers in early infancy and plantar calcaneal spurs in childhood are frequently seen in children with fibrodysplasia ossificans progressiva and may be a useful radiologic indicator for early diagnosis.

Keywords Calcaneal spur · Children · Double calcaneal ossification center · Early diagnosis · Fibrodysplasia ossificans progressiva · Radiography

Introduction

Fibrodysplasia ossificans progressiva is a genetic disorder of the connective tissues caused by activating mutations in the gene-encoding activin receptor IA/activin-like kinase 2 (ACVR1/ALK2), a bone morphogenetic protein (BMP) type I receptor [1]. Most affected individuals have a common mutation (c.617G > A, p.R206H). The clinical hallmark of fibrodysplasia ossificans progressiva is progressive heterotopic ossification of soft tissues, such as muscles, ligaments, tendons, fasciae and aponeuroses, which causes significant physical morbidity and may lead to early mortality. Traumatic injury and surgical intervention induce explosive heterotopic ossification in patients with fibrodysplasia ossificans progressiva. Early diagnosis is necessary to prevent additional trauma or iatrogenic harm [2].

Fibrodysplasia ossificans progressiva is associated with a variety of bone malformations as well as variant ossification. Awareness of the bone anomalies may facilitate prompt diagnosis. Malformations of the great toes are well-known and are the most prevalent indicators of this disorder [3]. Other anomalies include shortening of the first metacarpal bones and

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hypertrophy of the posterior element of the cervical spine [4]. Quantitative analyses of bone changes in the hand and cervical spine are also available [5]. Recently, we encountered two children with distinctive double ossification centers and plantar spurs in the calcaneus. Search of the database identified seven additional children with fibrodysplasia ossificans progressiva. We evaluate whether the presence of calcaneal changes may be commonly seen in patients with fibrodysplasia ossificans progressiva.

Materials and methods

Two children (cases 2 and 3 in Table 1) were seen clinically and subsequently presented and discussed in web case consultations. The online discussion enabled us to collect clinical data and foot radiographs from members of the Research Committee on Fibrodysplasia Ossificans Progressiva (Japan), who provide pediatric orthopedic services in four hospitals across Japan. Six Japanese children and one Indian child (5 males and 2 females) who underwent lateral feet radiography in infancy or early childhood were thus additionally enrolled. All nine children fulfilled the diagnostic criteria of fibrodysplasia ossificans progressiva, including deformities of the great toes, extraskeletal heterotrophic ossification, joint contractures, fusion of the cervical spine, broad femoral necks and osteochondroma-like lesions. Seven of the nine children underwent molecular testing, showing the common ACVR1/ALK2 mutation within the glycine/serine-rich regulatory (GS) domain (c.617G > A, p.R206H). All radiographs were examined by a single reader (G.N.) with 30 years' clinical experience in the field of skeletal dysplasias and specifically focused on calcaneal configurations.

Results

Fourteen lateral foot radiographs obtained between the ages of 1 month to 104 months were available for this study. Three children (cases 1, 2 and 4) underwent serial radiographic examinations in infancy and early childhood. We found two distinctive findings: 1) double calcaneal ossification centers and 2) plantar calcaneal spurs. These findings were bilateral and symmetrical. The radiologic findings and pertaining clinical information are summarized in Table 1.

Double calcaneal ossification centers

Four children (cases 1, 2, 3 and 4) showed double ossification centers in radiographs obtained in infancy (Figs. 1, 2, 3 and 4). Three of them (cases 2, 3 and 4) had a cleft separating the anterior two-thirds from the posterior third in the calcaneus. The posterior ossification centers were small, intermediate and large in size at ages 2, 4 and 10 months, respectively, partially fused with the anterior ossification centers at ages 4 and 10 months, and completely incorporated into the anterior ossifications at ages 12 months and 22 months. The remaining child (case 1) had a cleft separating the anterior third from the posterior two-thirds in the calcaneus. The anterior ossification center manifested as punctate multiple ossifications in early infancy, which evolved into double ossifications at age 11 months and completely coalesced at age 3 years.

Plantar calcaneal spur

Seven children showed a small spur from the plantar aspect of the posterior calcaneal body (Figs. 2, 3, 4 and 5). The spurs are pedunculated and projected posteriorly from the posterior

Table 1 Demographic, clinical, genetic and radiologic summaries of nine children with fibrodysplasia ossificans progressive

Case number	1				2		3	4		5	6	7	8	9
Sex	male				male		female	male		male	female	male	male	female
Current age	4Y8M				6Y		2Y9M	3Y2M		2Y	3Y5M	4Y6M	7Y7M	8Y8M
Ethnic background	Japanese				Caucasian		Turkish	Japanese		Indian	Japanese	Japanese	Japanese	Japanese
ACVR1 mutation	R206H				R206H		ND	R206H		R206H	ND	R206H	R206H	R206H
Great toe abnormalities	+				+		+	+		+	+	+	+	+
Other radiological findings of FOP	+				NA		NA	+		+	+	+	+	+
Ectopic ossification	+				-		-	-		-	-	-	+	+
Age at X-ray (months)	1	3	11	36	2	12	4	10	22	15	18	33	70	104
Double calcaneal ossification centers	punctate	punctate	+	-	+	-	+	+	-	-	-	-	-	-
Plantar calcaneal spur	-	-	-	-	+	+	+	+	+	+	-	+	+	+

NA not available , *ND* not determined

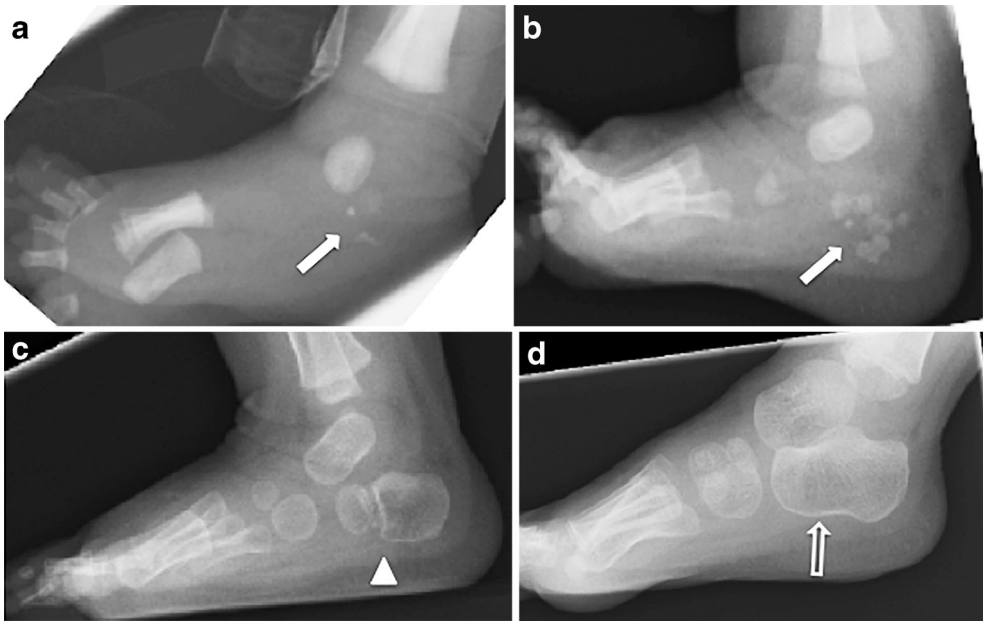

Fig. 1 Lateral foot radiographs in a boy with fibrodysplasia ossificans progressiva (case 1 in Table 1). Sequential radiographs at 1 months of age (**a**), 3 months of age (**b**), 11 months of age (**c**), and 3 years of age (**d**) demonstrate punctate multiple ossifications in early infancy (*solid arrows*), double ossification centers with a cleft separating the anterior third from the posterior two-thirds of the calcaneus (*arrowhead*), and normal calcaneal configuration after complete fusion of the ossification centers (*open arrow*)

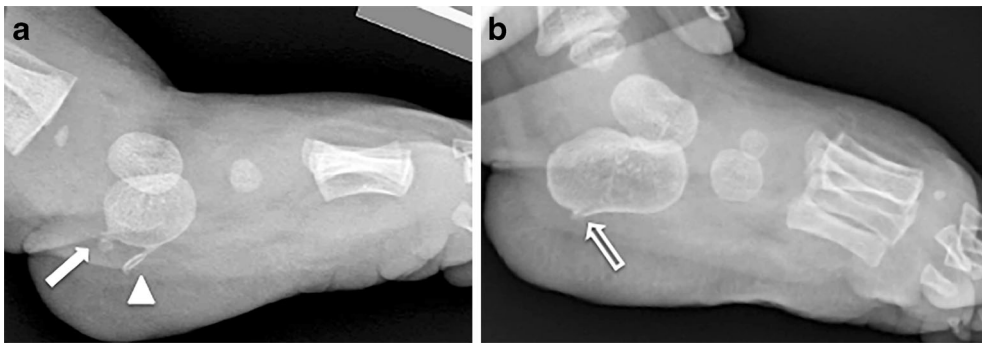

Fig. 2 Lateral foot radiographs in a boy with fibrodysplasia ossificans progressiva (case 2 in Table 1). Sequential radiographs at 2 months of age (**a**) and 12 months of age (**b**) show a small posterior ossification (*solid arrow*) and posteriorly pedunculated calcaneal spur (*arrowhead*), which became smaller in size with age (*open arrow*)

aspect of the anterior calcaneal ossification in infancy. They became sessile and projected inferiorly with age. In patient 2, the spur became smaller in size with age.

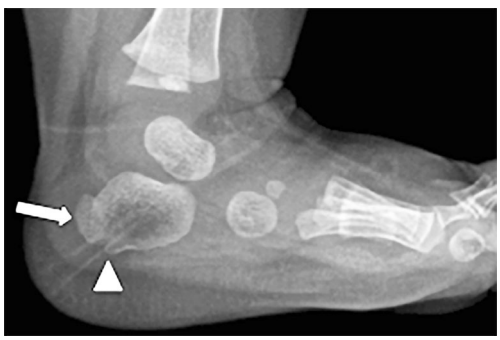

Fig. 3 Lateral foot radiograph in a girl with fibrodysplasia ossificans progressiva (case 3 in Table 1) at 4 months of age demonstrates double ossification centers (*arrow*) and plantar spur (*arrowhead*) of the calcaneus

Discussion

Our findings suggest that abnormal/variant calcaneal ossification, double calcaneal ossification and plantar spurs may be of diagnostic significance in patients with fibrodysplasia ossificans progressiva. These are congenital anomalies, which are perceptible in early infancy and can be a clue to the early diagnosis of this disorder, as are malformations of the great toes, shortening of the thumb and hypertrophy of the posterior element of the cervical spine. This finding is not exclusive of fibrodysplasia ossificans progressiva, as double calcaneal ossifications and plantar spurs are also described as rare developmental variations in normal children [6–10].

The normal double calcaneal ossification is sometimes referred to as bifid os calcis [8–10]. This variation has been reported in children with mild foot deformities, such as pes planovalgus, metatarsus varus and mild talipes equinovalgus. Bifid os calcis shows complete coalescence and remodeling of

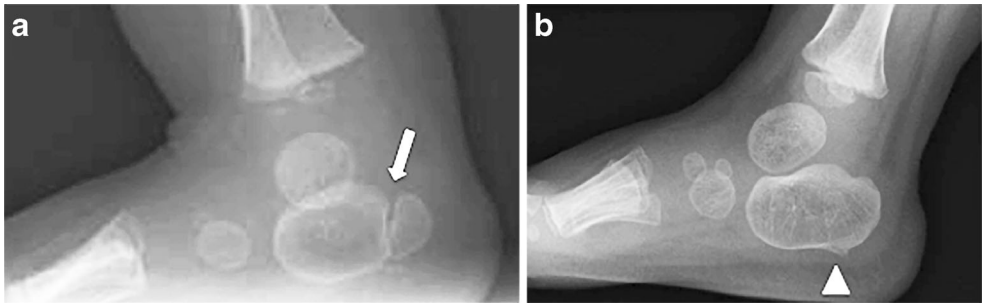

Fig. 4 Lateral foot radiographs in a boy with fibrodysplasia ossificans progressiva (case 4 in Table 1). Sequential radiographs at 10 months of age (**a**) and 22 months of age (**b**) reveal double ossification centers with a cleft separating the anterior two-thirds from the posterior third in the calcaneus (*arrow*) and an inferiorly projected small spur in the plantar calcaneus (*arrowhead*)

two separate ossifications during early childhood and typically shows a cleft separating the anterior third from the posterior two-thirds of the calcaneus. A patient with bifid os calcis reported by Ogden [9] showed punctate ossification centers in the anterior portion. In three children in our series, double calcaneal ossifications were associated with a cleft separating the anterior two-thirds from the posterior third of the calcaneus. However, the manifestation in the remaining one was similar to that in Ogden's case.

A duplicate/triplicate calcaneus was observed in specific skeletal dysplasias, including chondrodysplasia punctata, thanatophoric dysplasia and short rib polydactyly syndromes [11]. Double calcaneal ossifications with a cleft separating the anterior two-thirds from the posterior third of the calcaneus resemble those commonly seen in an infant with Larsen syndrome. Larsen syndrome is caused by heterozygous mutations in filamin B gene (*FLNB*). Filamen B is a cytoskeletal protein involved in a multicellular process [12]. Zheng et al. [13] demonstrated that *FLNB* mutant mice display ectopic mineralization in various cartilaginous elements, including carpal and tarsal bones, and this mutant phenotype is rescued by removing Runx2 through TGFβ-Smad pathway. Overexpression of the R206H mutant ACVR1, on the other hand, enhances Smad1/5 signaling. Molecular interactions between filamin B and Smad signaling in skeletal morphogenesis may lead to similar phenotypes of ossifications in the calcaneal region in Larsen syndrome and fibrodysplasia ossificans progressiva.

The normal plantar calcaneal spur is seen at the posterior two-thirds of the bone, tends to be bilateral and symmetrical, may point anteriorly, posteriorly or inferiorly, and disappears by 1 year of age. The normal calcaneal spur is morphologically indistinguishable from the late manifestation of the calcaneal spur in fibrodysplasia ossificans progressiva. However, the early pedunculated appearance in fibrodysplasia ossificans progressiva is not seen in the normal spur. In addition, the spur in fibrodysplasia ossificans progressiva persists in childhood.

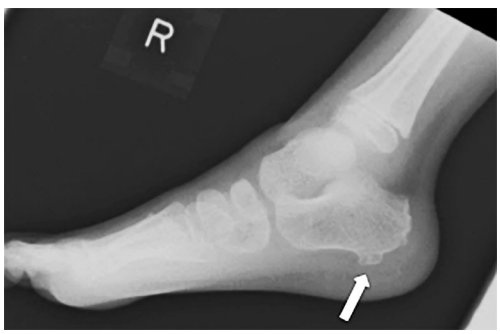

Fig. 5 Lateral foot radiograph in a boy with fibrodysplasia ossificans progressiva (case 8 in Table 1) at 5 years, 10 months of age shows a small spur from the plantar aspect of the posterior calcaneal body (*arrow*)

Conclusion

Double calcaneal ossification centers in early infancy and plantar calcaneal spurs in childhood may be significant radiologic findings useful for early diagnosis of fibrodysplasia ossificans progressiva.

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Compliance with ethical standards

Conflicts of interest None

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Follow-Up Study on Fetal CT Radiation Dose in Japan: Validating the Decrease in Radiation Dose

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OBJECTIVE. In 2011, we collected data on fetal CT radiation dose to determine the diagnostic reference level (DRL); however, continuous evaluation of the DRL is necessary. The hypothesis of this study is that the fetal CT radiation dose has decreased, and we predict a widespread use of iterative reconstruction (IR). We also predict that the national decrease in exposure is because of the DRL reported as a result of the previous national study.

MATERIALS AND METHODS. Various testing protocols from each site were summarized as part of the study results. The minimum, one-fourth (25th percentile), median, three-fourths (75th percentile), and maximum values were obtained for volume CT dose index ($CTDI_{vol}$), dose-length product (DLP), and scan length of 120 fetal CT examinations. The trends for IR usage and tube voltage were also investigated.

RESULTS. Compared to the results of the 2011 study (n = 119), the minimum, 25th percentile, median, and 75th percentile values for $CTDI_{vol}$ and DLP have decreased for the tabulated results in 2015 (n = 120). The 75th percentile value for $CTDI_{vol}$ was 4.9 mGy, which is 43% of the previous value. IR was used in 70% of the sites. The radiation dose was significantly lower among groups that used IR.

CONCLUSION. Four years passed between our initial survey on DRL and the present follow-up survey, and it appears that the previous report sufficiently fulfilled its objective and role in contributing to the decrease in DRL observed in this follow-up study.

Keywords: CT, diagnostic reference level, fetus, prenatal diagnosis, skeletal dysplasia

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According to an international classification issued in 2015 [1], there are 436 disease names, classified into 42 disease groups, and 364 responsible genes associated with genetic disease skeletal dysplasia. During the neonatal period, these diseases are found at a rate of two in 10,000 births, and half are lethal [2]. The recent widespread use of fetal ultrasound testing and improvement in diagnostic accuracy has led to the detection of short limbs and more cases of suspected skeletal dysplasia [3]. The advantage of ultrasound is that it can be used for pregnant women without involving x-ray irradiation. However, it is extremely difficult to detect, evaluate, and interpret the abnormal findings of skeletal structures using ultrasound, and a specific diagnosis cannot be made by the ultrasound operator without vast knowledge and experience in making such diagnoses. Unless the region that may contain the abnormalities is searched in detail during the scan, sufficient material for retrospective diagnosis cannot be obtained. This is the disadvantage of ultrasound testing that is based on real-time diagnosis.

However, the effectiveness of prenatal 3D CT for detecting fetal skeletal dysplasia has been previously reported, and 93% of pathognomonic abnormal findings confirmed after birth with simple x-ray were diagnosable with fetal skeletal CT. It was reported that the diagnosis determined by ultrasound was revised for about 60% of the cases after fetal CT [4].

Prenatal diagnosis using CT provides visual and clinical information to facilitate planning for a birthing method and selection of treatment by the various people involved, including the parents, family members, obstetrician, and perinatal care staff. It also allows the parents of the fetus to prepare themselves psychologically. Although there are many advantages, the risk of radiation exposure to the fetus and mother cannot be avoided because CT uses x-rays.

In 2011, we surveyed medical institutions in Japan that conduct fetal CT and collected data on CT conditions and radiation exposure; that study was published in 2014 [5].

From the data, we reported the estimated diagnostic reference level (DRL). To our knowledge, that report was the first and only fetal CT DRL study in the world. We decided to investigate DRL again in 2015 because 3 years and 9 months had passed since the initial survey was conducted. Periodic assessment of DRL every few years at each medical institution or at the national level is recommended [6]. Since the first study was conducted, CT equipment has seen further progress in the number of detectors, and high-performance CT equipment with 64 or more detectors are almost in common use. In addition, there are some reports in the pediatric radiology field that show an increase in popularity for using an iterative reconstruction (IR) protocol to reduce the radiation dose [7].

The hypothesis of this study is that the fetal CT radiation dose has decreased throughout the country compared with the nationwide study conducted in Japan in 2011 [5], and we predict the widespread usage of fetal CT with IR. We also predict that the national decrease in exposure is because of the DRL reported as a result of the previous national study.

Materials and Methods

Selection of Target Medical Institutions

This study was approved by the ethics board of the National Center for Child Health and Development and was conducted after approval of the institutional review board.

The 16 sites that were studied in the 2011 survey [5] are major facilities performing fetal CT in Japan and were included in this study. In addition, an Internet forum for fetal skeletal dysplasia based in Japan, the Japan Forum of Fetal Skeletal Dysplasia (with 53 registered users), was used to call for participation by medical institutions that did not participate in the previous study. Medical institutions that consulted the radiologist and obstetrician members of the forum were also selected as candidates. Furthermore, medical institutions that have presented results in past society meetings in Japan and overseas, or submitted journal papers, were sought and considered as candidates. As a result, 25 medical institutions were selected as study sites. These study sites were mailed a CD-ROM with the questionnaire sheet, together with a letter inquiring about their interest regarding participation. The following 22 medical institutions expressed interest: Hokkaido University Hospital; Aomori Prefectural Central Hospital; Miyagi Children's Hospital; Tohoku University Hospital; Yamagata University Hospital; Chiba Kaihin Municipal Hospital; Juntendo University Urayasu Hospital; National Center for Child Health and Development; Jikei University School of Medicine Hospital; Tokyo Women's Medical University Hospital; Fujita Health University Hospital; Nagara Medical Center; University Hospital, Kyoto Prefectural University of Medicine; Osaka Medical Center and Research Institute for Maternal and Child Health; Hyogo College of Medicine Hospital; Shikoku Medical Center for Children and Adults; Perinatal Medical Center, Ehime Prefectural Central Hospital; Kochi Health Sciences Center; Tokushima University Hospital; Hiroshima University Hospital; Perinatal Medical Center, Yamaguchi Prefectural Grand Medical Center; and Kurume University Hospital.

Preparation of the Work Sheet

The first page of the work sheet was to be entered by the obstetrician of the hospital, and the remaining pages were to be entered by the radiologic technologist or radiologist. Eight questions were listed on the first page. First, what is the category of your hospital (choice of three options: university hospital, perinatal medical center, or regional core hospital)? Second, was specific written consent for fetal CT obtained? Third, how many fetal CT examinations have been conducted in the past 3 years and 9 months? Fourth, at what range of fetal stage (weeks) was CT conducted and what was the mean number of weeks? Fifth, on the basis of the 2011 study [5], have there been any changes to the protocol? Sixth, if the protocol has been changed, has the diagnostic ability decreased as a result of lowering the dose? Seventh, is sedation used on the fetus when conducting fetal CT? Finally, do you know the approximate fetal CT radiation dose for your hospital?

The CT survey from the second page onward had entry sheets for four manufacturers: Toshiba, GE Healthcare, Siemens Healthcare, and Philips Healthcare. The sheet for the manufacturer of the equipment used for testing at each site was also filled out. If multiple pieces of CT equipment were used at one site, we requested that each piece was entered into separate work sheets. Because parameters are different depending on the manufacturer, the work sheet entry items tailored for each CT manufacturer were chosen by a subgroup of seven radiologic technologists selected from sites that frequently perform fetal CT. The staff in charge of setting imaging protocols for each manufacturer at each of the four CT manufacturers was requested to check for the appropriateness of the chosen work sheet items, and the revised items were distributed. The content of the work sheet differs for each company, but basic items include tube voltage, tube current, scan time, pitch, scan FOV, scan length, volume CT dose index ($CTDI_{vol}$), dose-length product (DLP), whether IR is used for scanning and 3D imaging, and the name and degree of IR protocol used. This questionnaire was prepared using Excel software (version 2013, Microsoft) and was distributed to each site.

The study started when the survey was sent on December 5, 2014, and results were collected by January 31, 2015. The study implementation period was 58 days.

Summary of the Collected Data on Fetal CT From the Sites

The frequency of usage for each piece of CT equipment and the protocol was summarized from the survey results. The CT parameters used at the sites were also compared among the results using the same protocol. The trend in results for the protocol that used the lowest dose and the protocol that used the highest dose was studied.

CT Exposure Evaluation Method

The minimum, one-fourth (25th percentile), median, three-fourths (75th percentile), and maximum values were obtained for the $CTDI_{vol}$, DLP, and scan length from the 139 fetal CT examinations collected in this 2015 study. The results were compared with the previous results in 2011 [5], and the changes over the 4 years were evaluated. In addition, the 75th percentile values of $CTDI_{vol}$ and DLP in this study were determined as the new DRL. The median value was compared for scan length.

Regarding usage of IR, the frequency of usage was studied and the difference in $CTDI_{vol}$ value was compared among cases with and without IR use.

Regarding tube voltage, the frequency of usage of 80, 100, and 120 kV was studied. The results were compared with those of the 2011 study [5]. In addition, the $CTDI_{vol}$ values for the frequently used voltages, 100 and 120 kV, were compared among the different protocols used. For statistical analysis of the data, an unpaired *t* test was used to test for significance (Excel version 2013, Microsoft).

Results

Response to Questions Addressed to the Obstetrician

Category of your hospital—The breakdown of the 22 sites was 13 university hospitals (59%), followed by six perinatal centers (27%), and three regional core hospitals (14%). However, there were inconsistencies in CT dose data on two sites. These sites were excluded and a total of 20 sites were used for analysis.

TABLE 1: Summary of 32 Fetal CT Protocols for Scanners Used at Each Survey Site

Manufacturer	Brand Name	Tube Voltage (kV)	Rotation Time (s)	Helical Pitch	Volume CT Dose Index (mGy)	Dose-Length Product (mGy·cm)	Iterative Reconstruction
GE Healthcare	Discovery 750HD (*n* = 3)	100 (*n* = 4)	0.4–1	0.5–1.4	0.5–9	18–353	ASiR (*n* = 3)
	LightSpeed 16 (*n* = 1)	120 (*n* = 1)					Veo (*n* = 2)
	LightSpeed VCT (*n* = 1)						
Siemens Healthcare	Sensation (*n* = 2)	120 (*n* = 10)	0.28–0.5	0.6–1.4	1.6–13	52–514	SAFIRE (*n* = 4)
	Definition (*n* = 8)						IRIS (*n* = 1)
Toshiba	Aquilion 16 (*n* = 4)	80 (*n* = 1)	0.3–0.75	0.8–1.5	1.8–27.5	55–943	AIDR3D (*n* = 6)
	Aquilion 64 (*n* = 4)	100 (*n* = 4)					
	Aquilion other (*n* = 4)	120 (*n* = 12)					QDS (*n* = 1)
	Aquilion ONE (*n* = 3)						
Philips Healthcare	iCT Elite (*n* = 1)	80 (*n* = 1)	0.4–0.75	0.6–0.9	1.6–7.9	70–281	IMR SoftTissue (*n* = 1)
	iCT (*n* = 1)	100 (*n* = 1)					iDose 4 (*n* = 1)
Summary of fetal CT protocols	*n* = 32	120 (*n* = 23)	0.28–0.75	0.5–1.5	0.5–27.5	18–943	Hybrid iterative reconstruction (*n* = 17)
		100 (*n* = 9)					Full iterative reconstruction (*n* = 2)

Note—ASiR = adaptive statistical iterative reconstruction, SAFIRE = sinogram-affirmed iterative reconstruction, IRIS = iterative reconstruction in image space, AIDR3D = adaptive iterative dose reduction 3D, QDS = quantum denoising software, IMR = iterative model reconstruction.

Specific written consent for fetal CT—Sixteen sites (80%) obtained specific written consent for conducting fetal CT.

Number of fetal CT examinations conducted in the past 3 years and 9 months—The number of cases per site during this time period ranged from one to 24 and there were 139 cases in total.

The range of and mean gestational week when CT was conducted—The range was 17–36 weeks' gestation, and the mean (± SD) was 30.1 ± 3.1 weeks.

Knowledge of the previous study report (2011), and whether the protocol has been changed—Six sites changed their protocol on the basis of the 2011 DRL (30%). Seven sites (35%) each answered that the study results did not affect their protocol or they did not know about the 2011 DRL.

Whether diagnostic ability decreased because of protocol change—No sites that changed their protocol answered that diagnostic ability decreased by lowering the dose.

Use of sedation on fetus during fetal CT—Two sites (10%) used sedation when conducting fetal CT, but the remaining 18 sites (90%) did not use sedation. One site that used sedation answered "walking, use of Diazepam in some cases," and the other site did not give a detailed response.

Knowledge of an approximate fetal CT radiation dose at own site—Obstetricians at 17 sites (85%) answered that they knew the approximate fetal CT radiation dose at their own site.

Summary of Collected Data on Fetal CT From Each Site

Table 1 summarizes the survey results about the 32 CT protocols used by the sites for each scanner manufacturer. The most common scanner manufacturer among the survey sites was Toshiba; however, no obvious difference of parameter setting was identified among CT vendors.

There was a wide range of radiation doses as measured by $CTDI_{vol}$ and DLP, and the lowest scanning condition was a $CTDI_{vol}$ of 0.5 mGy with the full IR method (Veo, GE Healthcare), even though full IR was used for only 10% of the protocols. This is somewhat surprising, because the maximum values of $CTDI_{vol}$ and DLP (27.5 mGy and 943 mGy·cm, respectively) were 50 times as large as the minimum settings (0.5 mGy and 18 mGy·cm, respectively). Approximately 68% of CT protocols were performed using a tube voltage of 120 kV.

CT Exposure Evaluation

Among the 139 fetal CT examinations performed at the study sites during the study period, inquiries were made to the staff in charge of setting imaging protocols for each manufacturer regarding incomplete data submitted from the sites to recover and ensure consistency in the data. However, inconsistencies in data on $CTDI_{vol}$ and DLP for 19 cases could still not be resolved. These data were excluded, and a total of 120 cases were used for analysis.

Evaluation and Change of Volume CT Dose Index, Dose-Length Product, and Scan Length

The comparison of values for $CTDI_{vol}$, DLP, and scan length in 2011 and 2015 is

TABLE 2: Comparison of Volume CT Dose Index ($CTDI_{vol}$), Dose-Length Product (DLP), and Scan Length Between 2011 and 2015

	$CTDI_{vol}$ (mGy)[a]		DLP (mGy·cm)[a]		Scan Length (mm)[b]	
Measurement	2011	2015	2011	2015	2011	2015
Maximum	23.1	27.5	1025.6	943.5	476	520
75th percentile	11.3	4.9	382.6	176.4	356	341
Median	7.7	3.2	276.8	104.3	319	313
25th percentile	3.7	2.4	122.3	84.8	295	287
Minimum	2.1	0.5	69	18.3	190	133

[a] $p < 0.01$.
[b] $p > 0.05$.

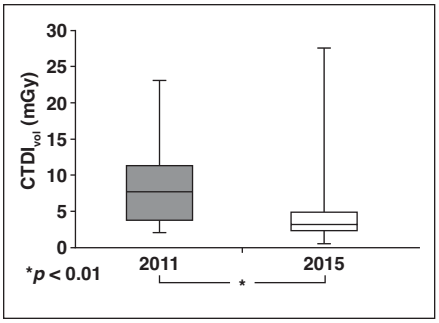

Fig. 1—Boxplot of volume CT dose index ($CTDI_{vol}$) values in 2011 and 2015. Boxes denote ranges, lines in boxes denote medians, and vertical lines and whiskers denote 95% CIs.

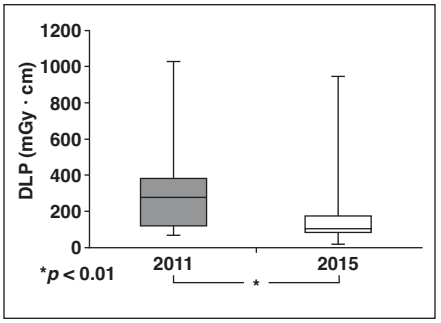

Fig. 2—Boxplot of dose-length product (DLP) values in 2011 and 2015. Boxes denote ranges, lines in boxes denote medians, and vertical lines and whiskers denote 95% CIs.

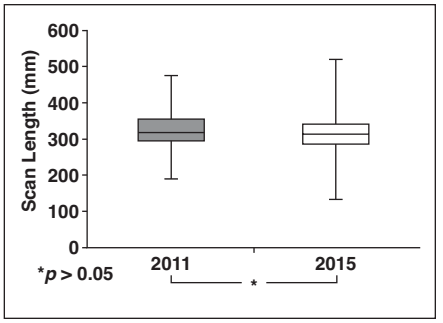

Fig. 3—Boxplot of scan length values in 2011 and 2015. Boxes denote ranges, lines in boxes denote medians, and vertical lines and whiskers denote 95% CIs.

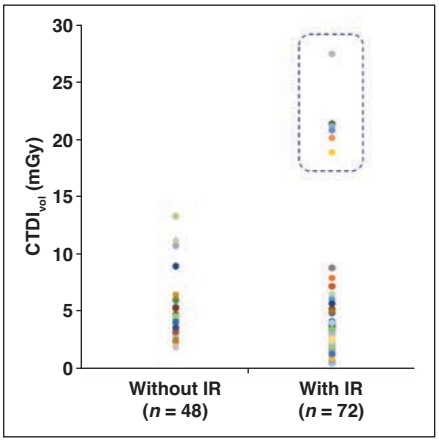

Fig. 4—Comparison of median volume CT dose index ($CTDI_{vol}$) between CT performed with and without iterative reconstruction (IR). If sites surrounded by dashed line are excluded, $CTDI_{vol}$ values for sites using IR are lower than for those that do not use IR, with statistically significant difference between groups ($p < 0.01$).

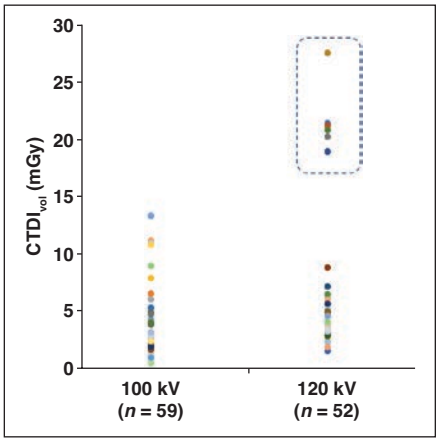

Fig. 5—Comparison of median volume CT dose index ($CTDI_{vol}$) values at 120 and 100 kV. In contrast to results for iterative reconstruction, results for tube current did not show significant difference in $CTDI_{vol}$ even when sites surrounded by dashed line were excluded.

shown in Table 2, and boxplots are shown in Figures 1–3. Compared with the study in 2011 (n = 119), the minimum, median, 75th percentile, and 25th percentile values of $CTDI_{vol}$ and DLP for the tabulated results in 2015 (n = 120) were lower, although some sites had a higher maximum value than that of the previous study. The 75th percentile value of $CTDI_{vol}$ that is the DRL for this study was 4.9 mGy, and this was 43% of the previous value of 11.3 mGy. During this period, a 57% reduction in the radiation dose was found (Fig. 1). Similar to $CTDI_{vol}$, the 75th percentile value for DLP was 176.4 mGy·cm, and this was 46% (about half) of the previous value of 382.4 mGy·cm. The differences were statistically significant ($p < 0.01$) (Fig. 2). On the other hand, the scan range was almost the same as in the previous study, and although the data included over 100 cases, the median was different by only 6 mm, and the data were considered to be accurate. It was found that the decrease in DLP was not caused by a shortening of the length (Fig. 3).

In this study, 14 of 20 sites (70%) used IR. Among the 14 sites that used IR, six (43%) did not use the method at the beginning of the study period but started to incorporate it during the study period. As shown in Figure 4, a comparison of $CTDI_{vol}$ between CT with IR (n = 72) and CT without IR (n = 48) shows that it is lower in the group that uses IR than the group that does not use IR, although one site had set a significantly higher radiation dose than the others (section surrounded by the dashed line in Fig. 4). If the site surrounded by the dashed line is excluded, the $CTDI_{vol}$ for sites using IR compared with those that do not use IR is statistically significantly lower ($p < 0.01$). The group that uses IR had a significantly lower radiation dose setting compared with the group that did not (Table 3).

Tube voltage was 120 kV in 52 cases, 100 kV in 59 cases, and 80 kV in nine cases, with 100 kV the most frequently used voltage. Six sites had the voltage fixed at 120 kV, whereas the other 14 sites used a tube voltage lower than 120 kV.

As with IR, comparison of $CTDI_{vol}$ values by tube current (120 vs 100 kV) showed that the median $CTDI_{vol}$ values for 120 and 100 kV were 3.3 and 2.5 mGy, respectively. As shown in Figure 5, the tube voltage was set at a significantly higher radiation dose in one

TABLE 3: Relationships Between Volume CT Dose Index ($CTDI_{vol}$) and Iterative Reconstruction (IR) and Between $CTDI_{vol}$ and Tube Voltage

	$CTDI_{vol}$ With and Without IR (mGy)[a]		$CTDI_{vol}$ at Different Tube Voltages (mGy)[b]	
Measurement	With IR	Without IR	120 kV	100 kV
Maximum	8.8	13.4	8.8	13.4
75th percentile	3.5	5	4.8	4.1
Median	2.5	3.3	3.3	2.5
25th percentile	2	3.2	3.2	2
Minimum	0.5	1.8	1.6	0.5

[a] $p < 0.01$.
[b] $p > 0.05$.

site in the group that used IR (section surrounded by a dashed line in Fig. 5). In contrast to the results for IR, the results for tube current did not show a significant difference in $CTDI_{vol}$ even when the site surrounded with the dashed line was excluded.

Discussion

From the response to the questions answered by the obstetricians, the following situation was revealed. In Japan, 86% of fetal CT examinations were performed at university hospitals or public perinatal centers that act as the center for medical care in that region. It is assumed that obstetricians and gynecologists at local clinics who detect short limbs by fetal ultrasound send a referral to obstetricians or fetal examination specialists for retesting by ultrasound with higher accuracy, and then the case is indicated for CT if necessary.

In Japan, 80% of the sites obtained specific written consent for fetal CT with informed consent from the parents regarding fetal radiation exposure.

The mean timing for the CT was 30.1 ± 3.1 weeks' gestation. This is thought to be the result of a recommendation by the leading academic research group on fetal CT in Japan, the Japan Forum of Fetal Skeletal Dysplasia, which recommends testing at around 30 weeks. This recommendation is based on several reasons, including the fact that the effect of radiation on the CNS is less of a concern during the third trimester of pregnancy [8] and that depiction of the skeletal structure is clearer compared with the early stages of pregnancy because of fetal growth. The result was almost the same as the previous study [5] (mean, 30.2 ± 2.6 weeks) with essentially no change. It is understandable that the sites that perform CT are actually following the recommendations of the Japan Forum of Fetal Skeletal Dysplasia.

Thirteen sites (65%) were aware of the DRL results from the 2011 study [5], and six sites (30%) had changed their protocol on the basis of the 2011 DRL. On the other hand, seven sites (35%) answered that they were not aware of the study results. DRL was proposed in the 1980s to optimize simple x-ray radiation dose and it was introduced as a method for optimizing CT radiation dose in the 1990s [7, 9]. It is considered that there is high awareness of DRL in the sites performing fetal CT, and DRL is correctly functioning as a regulatory pressure on the entire community as intended. No sites responded that a lower dose resulting from protocol changes led to decreased diagnostic ability, and this further reinforces that these scanning conditions are appropriate.

It was revealed that 90% of sites did not sedate the fetus for fetal CT. In the previous survey, one site used pancuronium (Mioblock, MSD) to suppress fetal movement, but this response was not found in the current study. It is considered that sedation is not necessary for fetal CT.

Obstetricians at 17 sites (85%) knew the approximate fetal CT radiation dose of their site when they order the scan. According to a literature search, it has been reported in a past survey that only 16% of residents in a radiology department knew the radiation dose for abdominal CT [10]. The obstetricians at sites performing fetal CT may be aware of the dose because they are required to explain the dose to patients. This may be inaccurate because the specific number was not asked, but compared with past reports, obstetricians who are involved in fetal CT are considered to have a fairly high awareness of CT exposure.

This study has revealed that since the 2011 study [5], the DRL for fetal CT in Japan has been reduced by half, a result that was statistically significant. This may be due to three factors. One is the first-ever proposal of fetal CT DRL in Japan from the study results in 2011. The results were widely reported at fetal CT forums and research groups that were active in performing fetal CT. A total of 65% of the sites studied in this analysis answered that they were aware of the results, and, furthermore, 30% of sites responded that they had lowered their dose setting on the basis of the DRL of the previous study. This is the result of DRL fulfilling its correct function, and it could be evaluated as an extremely significant result for managing CT radiation exposure in Japan. It is considered that DRL should be studied every few years and, indeed, this study was conducted at the appropriate time. We should conduct another national survey in 3–4 years and confirm the decrease in DRL as a community.

The next factor is the decrease in dose due to widespread use of IR. In the previous 2011 study [5], only one site of 16 (6%) used IR, but in this study, there was a significant increase with 14 of 20 sites (70%) using IR. This is considered to be due to the recent recognition of IR in all CT scans, including those of children [11] and adults, and the experience of the scanner, who can easily enhance image quality by changing the console setting. Therefore, among the 14 sites that used IR, the fact that six sites (43%) did not use IR at the beginning of the study but introduced IR later during the study period is considered to be the result of recognizing IR's usefulness in fetal CT.

The site that performs scans with the lowest exposure ($CTDI_{vol}$, 0.5 mGy) in this study does not use hybrid type IR but has introduced full IR (Veo, GE Healthcare) to aim for lower exposure. There are only a few reports on using full IR for fetal CT [12], but a decrease in fetal CT radiation dose would be possible if the full IR method becomes widespread, leading to a further decrease in DRL throughout Japan.

Similarly, this study found that many sites used low tube voltage. No significant difference in $CTDI_{vol}$ was found between different protocols using 120 or 100 kV, but the use of low tube voltage at these sites may be the result of awareness of lowering exposure and an adjustment to avoid an increase in unnecessary tube current. This is thought to be due to a change in techniques and awareness among the technicians in charge of CT machines in the clinical field.

In this way, radiation exposure of fetal CT has been optimized, but as shown in Figures 4 and 5, there are still sites with conditions leading to high radiation doses, and further education on DRL is required.

On the other hand, a limitation of this and previous studies was that only the radiation dose to set DRL was studied, and we did not collect data on image quality. Focusing on decreasing the dose may lead to a failure to maintain sufficient diagnostic quality for images. Goske et al. [13] have published their thoughts regarding the diagnostic reference range as a method to maintain both an appropriate radiation dose management for CT and diagnostic image quality. They suggest that the upper limit for radiation exposure should be the 75th percentile value and the lower limit should be the 25th percentile value to maintain image quality, and the appropriate scan condition should fall within this range. If this is applied to the results of this study, the appropriate dose for fetal CT would be a $CTDI_{vol}$ between 2.5 and 5 mGy (median, 3 mGy). In cases without full IR, this range for diagnostic reference range is considered to be appropriate.

A significant reduction in radiation exposure is possible if fetal CT is used only for observation of skeletal structure of the fetus, but it is necessary for each medical institution to consider the characteristics of IR for their fa-

cility's CT equipment and to perform a CT examination with sufficient quality for diagnosis.

In summary, since the previous DRL survey was conducted [5], the original objective and role for DRL that the International Commission on Radiological Protection [14] had proposed has been sufficiently reached. In addition, widespread use of IR and low tube voltage has helped in managing the radiation dose throughout Japan.

The objective of CT is to deliver an accurate diagnosis when skeletal dysplasia is suspected after careful fetal ultrasound testing and to determine the strategy for perinatal patient care. From this study, it was heartening to find that 85% of obstetricians who order CT scans were aware of the approximate CT radiation dose at their site.

We intend to conduct another survey in 4 years to confirm whether there is a further decrease in DRL. In addition, compared with 2011, there is concern that image quality may have been affected by the decrease in dose. Ideally, the next study would also include an evaluation of image quality.

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ORIGINAL ARTICLE

Influenza 2014–2015 among pregnant Japanese women: primiparous vs multiparous women

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Abstract This study was performed to determine whether multiparous pregnant women are prone to influenza. A questionnaire survey was conducted at 19 centres located throughout Japan, targeting all 6,694 postpartum women within 7 days after birth before leaving the hospital. All women gave birth during the study period between March 1, 2015, and July 31, 2015. Data regarding vaccination and influenza infection in or after October 2014, age, previous experience of childbirth, and number and ages of cohabitants were collected. Seventy-eight percent ($n = 51{,}97$) of women given questionnaires responded. Of these, 2,661 (51 %) and 364 (7.0 %) women reported having been vaccinated and having contracted influenza respectively. Multiparous women had a higher risk of influenza regardless of vaccination status (8.9 % [121/1362] vs 5.7 % [74/1299], relative risk [95 % confidence interval], 1.80 [1.36 to 2.38] for vaccinated and 9.3 % [112/

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1198] vs 4.3 % [57/1328], 2.18 [1.60 to 2.97] for unvaccinated women) compared to primiparous women. The risk of influenza increased with increasing number of cohabitants: 4.8 % (100/2089), 7.5 %, (121/1618), 9.0 %, (71/785), and 10.4 % (58/557) for women with 1, 2, 3, and ≥4 cohabitants respectively. Family size is a risk factor for influenza infection in pregnancy.

Introduction

Pregnant women are at increased risk of severe influenza-related complications [1, 2]. Despite accounting for approximately 1.0 % of the total population, pregnant women accounted for 5, 7.5, and 8.3 % of hospitalised cases in Canada, the UK, and Brazil respectively [3], and 9.1 % of 722 patients requiring treatment at an intensive care unit (ICU) in Australia and New Zealand [4] during the previous influenza H1N1 pandemic in 2009.

A meeting designed to integrate scientific evidence and expert opinion [5] in 2008 in the USA concluded that pregnant women should be considered a high-priority group for receipt of vaccine, and that increased seasonal influenza vaccine coverage may improve vaccine uptake in a pandemic [5]. The WHO recommends that pregnant women should be given the highest vaccination priority [6]. Therefore, it is important to determine the attitudes of pregnant women toward vaccination. During the 2013–2014 influenza season in Japan, approximately half of all pregnant Japanese women received influenza vaccination, which reduced the risk of influenza infection by 35 % among these women [7].

This previous investigation indicated multiparous pregnant women had an approximately twofold higher risk of influenza infection compared with primiparous pregnant women regardless of vaccination status in any age category [7].

The present multicentre questionnaire survey was conducted among postpartum Japanese women who gave birth during a 5-month period between March 1, 2015, and July 31, 2015, to determine the reproducibility of this phenomenon, i.e., that multiparous pregnant women are more vulnerable to influenza than primiparous pregnant women, and to test the hypothesis that the risk of influenza in pregnant women is related to number of cohabitants.

Materials and methods

This multicentre observational study was conducted with the approval of the Institutional Review Boards of Hokkaido University Hospital (No. 014-0265) and each of the following 19 participating hospitals widely dispersed throughout Japan: Kagoshima City Hospital (designated as A in Fig. 1), Fukuda Hospital (B), Nagasaki University Hospital (C), Hiroshima University Hospital (D), Osaka Medical Center and Research Institute for Maternal and Child Health (E), Rakuwakai Otowa Hospital (F), Mie Chuo Medical Center (G), Toyama University Hospital (H), Kitasato University Hospital (I), Nippon Medical School Tama-Nagayama Hospital (J), Shirota Obstetrical and Gynecological Hospital (K), Showa University Northern Yokohama Hospital (L), Showa University Hospital (M), University of Tsukuba Hospital (N), Jichi Medical University Hospital (O), Hakodate Central General Hospital (P), JCHO Hokkaido Hospital (Q), Sapporo Toho Hospital (R), and Hokkaido University Hospital (S).

In Japan, women usually remain at obstetric facilities for 4–8 days after giving birth. We conducted an anonymous questionnaire study (Table 1) among all postpartum women who gave birth at and after gestational week 22 and within 7 days after delivery before leaving the obstetric facility during the study period from March 1, 2015, to July 31, 2015. Therefore, the majority of these women conceived in or before October 2014.

All data are presented as the median (range). For statistical analysis of categorical data, the χ^2, Fisher's exact test, or Mann–Whitney *U*-test was applied for comparison of medians. The statistical software package StatView 5.0 for Macintosh (SAS Institute Inc., Cary, NC, USA) was used for data analysis. In all analyses, $P<0.05$ was taken to indicate statistical significance.

Results

During the 5-month study period, a total of 6,694 women, including 3,475 primiparous and 3,219 multiparous women, gave birth on or after gestational week 22 at the 19 participating hospitals (Table 2). Of these, 5,197 (78 %) women, consisting of 2,635 (76 %) of the 3,475 primiparous women and 2,562 (80 %) of the 3,219 multiparous women, responded to the questionnaire and participated in this study (Table 2). The 5,197 women corresponded to approximately 1.2 % of all expected 440,000 maternities occurring in the study period in Japan, which has a population of approximately 130,000,000. Younger primiparous women aged <30 years were less likely to participate in this study (Table 2). The response rate at each hospital is shown in the legend for Fig. 1.

Influenza infection rate in primiparous vs multiparous women

A total of 364 women (7.0 %) reported having contracted influenza during the current pregnancy (Table 3). The infection rate was significantly higher for multiparous than for primiparous women (9.1 % [233/2562] vs 5.0 % [131/2635], $P=0.0000$; relative risk [RR] with 95 % confidence interval

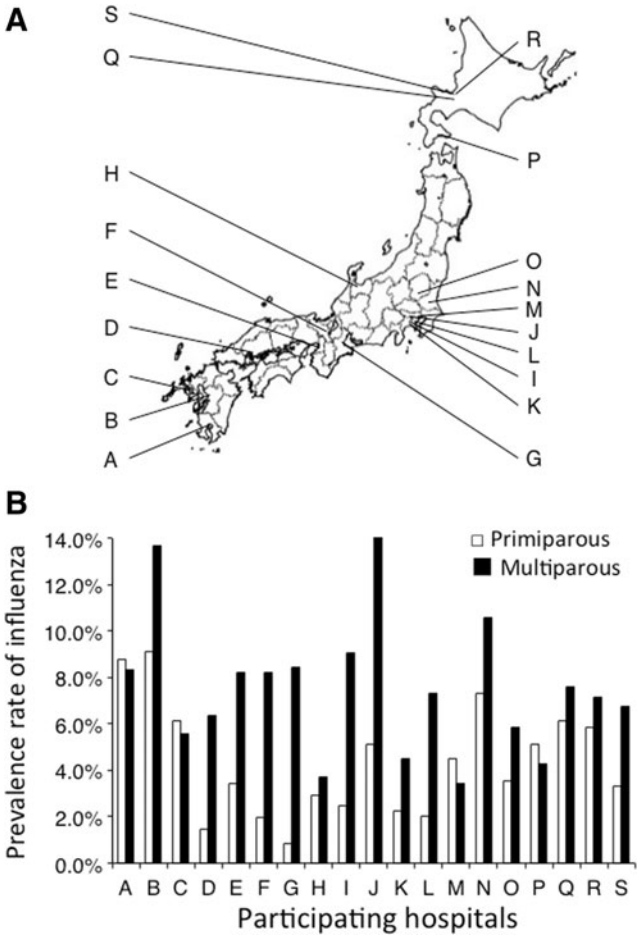

Fig. 1 Influenza infection rates among primiparous vs multiparous women in 19 hospitals located throughout Japan. **a** Locations of 19 hospitals that participated in this study on a map of Japan. **b** Influenza infection rates at the 19 hospitals. Although the infection rate was slightly higher among primiparous than multiparous women at 4 (21 %) hospitals (*A*, *C*, *M*, and *P*), the median (range) infection rate at the 19 hospitals was significantly higher for multiparous than for primiparous women (7.5 % [3.4–13.7 %] vs 4.0 % [0.8–13.6 %] respectively, $P = 0.00338$). The influenza infection rates at these hospitals for primiparous vs multiparous women were as follows: 8.8 % (5/57) vs 8.3 % (4/48) at hospital *A*, 9.1 % (46/505) vs 13.7 % (85/622) at *B*, 6.2 % (4/65) vs 5.6 % (3/54) at *C*, 1.4 % (1/69) vs 6.4 % (3/47) at *D*, 3.4 % (10/291) vs 8.2 % (24/293) at *E*, 2.0 % (1/51) vs 8.2 % (5/61) at *F*, 0.8 % (1/120) vs 8.2 % (8/95) at *G*, 2.9 % (1/34) vs 3.7 % (1/27) at *H*, 2.5 % (4/163) vs 9.1 % (14/154) at *I*, 5.1 % (3/59) vs 18.3 % (13/71) at *J*, 2.3 % (2/88) vs 4.5 % (5/111) at *K*, 2.0 % (4/200) vs 7.3 % (14/191) at *L*, 4.5 % (9/200) vs 3.4 % (5/146) at *M*, 7.3 % (16/218) vs 10.6 % (20/189) at *N*, 3.6 % (6/168) vs 5.8 % (10/171) at *O*, 5.1 % (2/39) vs 4.3 % (2/47) at *P*, 6.1 % (6/98) vs 7.6 % (7/92) at *Q*, 5.8 % (7/120) vs 7.1 % (6/84) at *R*, and 3.3 % (3/90) vs 6.8 % (4/59) at hospital *S* respectively. The response rates to our questionnaire among primiparous vs multiparous women at these hospitals were as follows: 49 % (57/117) vs 36 % (48/134) at hospital *A*, 100 % (505/505) vs 100 % (622/622) at *B*, 96 % (65/68) vs 84 % (54/64) at *C*, 100 % (69/69) vs 100 % (47/47) at *D*, 87 % (291/333) vs 88 % (293/334) at *E*, 86 % (51/59) vs 80 % (61/76) at *F*, 88 % (120/136) vs 90 % (95/106) at *G*, 38 % (38/89) vs 38 % (27/72) at *H*, 82 % (163/200) vs 81 % (154/190) at *I*, 54 % (59/110) vs 59 % (71/121) at *J*, 67 % (88/132) vs 80 % (111/138) at *K*, 60 % (200/335) vs 76 % (191/250) at *L*, 71 % (200/280) vs 66 % (146/220) at *M*, 93 % (218/235) vs 94 % (189/201) at *N*, 88 % (168/191) vs 94 % (171/181) at *O*, 61 % (39/64) vs 64 % (47/73) at *P*, 82 % (98/120) vs 81 % (92/114) at *Q*, 36 % (120/336) vs 39 % (84/214) at *R*, and 94 % (90/96) vs 95 % (59/62) at *S*, respectively

[95%CI], 1.83 [1.49 to 2.25]). Indeed, the infection rate was higher in multiparous than in primiparous women at 15 (79 %) of the 19 hospitals (Fig. 1) in which the median (range) infection rate was significantly higher for multiparous than for primiparous women (7.5 % [3.4–13.7 %] vs 4.0 % [0.8–13.6 %], $P = 0.0034$).

Vaccination coverage rate and effect of vaccination on influenza infection

The overall vaccination coverage rate was 51 % (2,661/5,197) (Table 3) and did not differ markedly between primiparous and multiparous women (49 % [1,299/2,635] vs 53 % [1,362/2,562] respectively). Maternal age affected vaccination coverage—women aged <25 years received vaccination significantly less often than those aged ≥25 years (see legend for Fig. 2). Vaccines against influenza used in Japan in 2014–2015 did not work to reduce the number of pregnant women with influenza (Table 3, Fig. 2). Overall infection rate did not differ significantly between those with and without vaccination (7.3 % [195/2,661] vs 6.7 % [169/2,526] respectively) (Table 3). The infection rate did not differ significantly between those with and without vaccination among primiparous (5.7 % [74/1,299] vs 4.3 % [57/1,328] respectively) as well as multiparous women (8.9 % [121/1,362] vs 9.3 % [112/1,198] respectively). Thus, multiparous women had a higher risk of influenza regardless of vaccination status compared to primiparous women (8.9 % vs 5.7 %; RR [95 % CI], 1.80 [1.36 to 2.38] for vaccinated women and 9.3 % vs 4.3 %; 2.18 [1.60 to 2.97] for unvaccinated women).

There was no consistent association between maternal age and the risk of influenza infection (Fig. 2). The median (range) vaccination coverage rate among 19 hospitals was 49 % (27–72 %) vs 52 % (31–70 %) for primiparous vs multiparous women. No significant correlation was seen between vaccination coverage rates and influenza infection rates (data not shown).

Effect of cohabitant number on influenza infection rate (Fig. 3)

As expected, the number of cohabitants was significantly greater for multiparous than primiparous women (2 [0–9] vs 1 [0–11], respectively, $P < 0.0001$). The influenza infection rate increased with increasing number of cohabitants among pregnant women with at least one cohabitant (Fig. 3). The presence of at least one child aged 1–17 years consistently increased the risk of influenza in pregnancy at any family size. Overall infection rate was higher for those with at least one

Table 1 Questionnaire form given to postpartum women with deliveries during the study period (March 1, 2015, to July 31, 2015)

Q1: Please specify your age in parenthesis.
I am (___) years old.
Q2: Was the current childbirth your first experience of childbirth?
□Yes, □No
Q3: Were you vaccinated against influenza on or after October 2014?
□Yes, □No
Q4: Please specify number of cohabitants according to their age in parentheses.

- Infants aged less than 1 year: ( ) persons
- Children aged 1 to 17years: ( ) persons
- adults aged 18 years of more: ( ) persons

Q5: What was your job that accounted for most time of your pregnancy?
□ mainly housekeeping
□ mainly jobs done outside your home
Q6: Did you contract influenza during the current pregnancy?
□Yes, □No
The following questions are for women answering "Yes" in response to Q6

Q7: What was the type of influenza?
□A, □B, □Unknown
Q8: Did you receive antiviral agent for the treatment of influenza?
□Yes, □No

child aged 1–17 years than in those without such children (9.0 % [226/2499] vs 5.1 % [138/2698], $P < 0.0001$). Although the median cohabitant number was 2 for both women with and without influenza (Table 3), the distribution of number of cohabitants differed significantly between women with and without influenza ($P < 0.0001$). The number of women with ≥3 cohabitants was significantly greater for those with than without influenza (35 % [129/364] vs 25 % [1,213/4,833], respectively, $P < 0.0001$).

Risk of influenza infection in women whose main occupation was housekeeping during the current pregnancy

Housekeeping was the main occupation in 49 % (2,541) of the 5,197 women (Table 3). Neither maternal age nor number of cohabitants differed significantly between those who worked within and outside the home (33.0 [15–48] vs 32.5 [16–48] years for maternal age, respectively; 2 [0–11] vs 2 [0–9] for

Table 2 Maternal age distribution among all 6694 candidates and 5197 respondents

	All candidates (primiparous)	Respondents (primiparous)
Maternal age (years)		
≤19	83 (70), [1.2 % (2.0 %)]	56 (50), [1.1 % (1.9 %)]
20–29	2,036 (1313), [30.4 % (37.8 %)]	1,437 (933), [27.7 % (35.4 %)]
30–34	2,200 (1049), [32.9 % (30.2 %)]	1,728 (791), [33.2 % (30.0 %)]
35–39	1,793 (749), [26.8 % (21.6 %)]	1,456 (607), [28.0 % (23.0 %)]
≥40	582 (294), [8.7 % (8.5 %)]	515 (251), [9.9 % (9.5 %)]
Unknown	0 (0), [0.0 % (0.0 %)]	5 (3), [0.1 % (0.1 %)]
Overall	6,694 (3475), [100 % (100 %)]	5,197 (2635), [100 % (100 %)]

Percentages of all women (primiparous women) are indicated in square brackets

Table 3 Demographic characteristics of 5,197 participants

	Influenza infection during current pregnancy	
	Yes	No
No. of women		
Primiparous	131 (36.0 %)	2,504 (51.8 %)
Multiparous	233 (64.0 %)	2,329 (48.2 %)
Unknown	0 (0 %)	0 (0 %)
Total	364	4,833
Maternal age (years)		
Median (range)	32.0 (20–45)	33.0 (15–48)
≤19	0 (0 %)	56 (1.2 %)
20–34	243 (66.8 %)	2,922 (60.5 %)
35–39	89 (24.4 %)	1,367 (28.3 %)
≥40	32 (8.8 %)	483 (10.0 %)
Unknown	0 (0 %)	5 (0.1 %)
No. of cohabitants		
Median (range)	2.0 (0–8)	2.0 (0–11)
0	14 (3.8 %)	133 (2.8 %)
1	100 (27.5 %)	1,990 (41.2 %)
2	121 (33.2)	1,497 (31.0)
3	71 (19.5)	714 (14.8)
≥4	58 (15.9 %)	499 (10.3 %)
Unknown	0 (0 %)	0 (0 %)
Job		
Housekeeping	150 (41.2 %)	2,391 (49.5 %)
Outside home	212 (58.2 %)	2,404 (49.7 %)
Unknown	2 (0.5 %)	38 (0.8 %)
Vaccination		
Yes	195 (53.6 %)	2,466 (51.0 %)
No	169 (46.4 %)	2,357 (48.8 %)
Unknown	0 (0 %)	10 (0.2 %)
Type of influenza		
A	270 (74.2 %)	
B	36 (9.9 %)	
Unknown	58 (15.9 %)	
Use of antiviral agent		
Yes	282 (77.5 %)	2 (0.0 %)
No	74 (20.3 %)	4,831 (100.0 %)
Unknown	8 (2.2 %)	0 (0 %)

number of cohabitants respectively). However, women with housekeeping had a significantly reduced risk of influenza by approximately 27 % (5.9 % [150/2541] vs 8.1 % [212/2616] (Table 3); RR [95%CI], 0.73 [0.60 to 0.89]).

Discussion

To our knowledge, this is the first study demonstrating family size as a risk factor for influenza infection in pregnancy. The

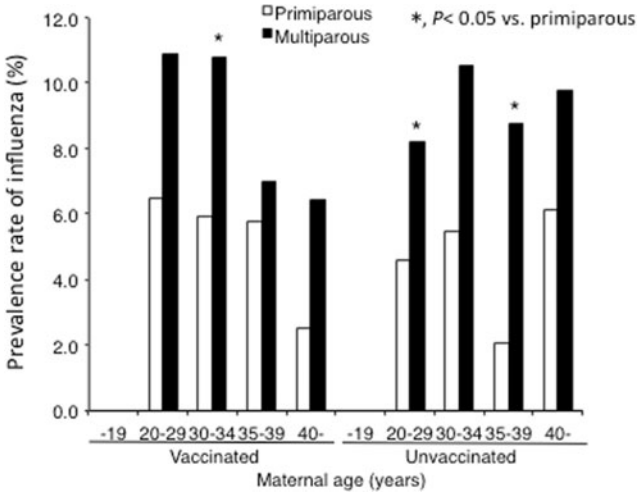

Fig. 2 Influenza infection rates according to maternal age and vaccination status among primiparous vs multiparous women. For primiparous vs multiparous women, overall vaccination coverage rate was 49 % (1299/2635) vs 53 % (1362/2562) respectively, and was 24 % (12/50) vs 33 % (2/6) for those aged <20 years, 29 % (70/239) vs 26 % (25/97) for 20–24 years, 52 % (361/694) vs 46 % (186/407) for 25–29 years, 53 % (422/791) vs 52 % (491/937) for 30–34 years, 52 % (313/607) vs 61 % (517/849) for 35–39 years, and 47 % (119/251) vs 53 % (140/264) for those aged ≥40 years respectively. Influenza was consistently more prevalent for multiparous than primiparous women in all age categories regardless of vaccination status. No influenza infection occurred in teenage pregnant women (0.0 % [0/50] vs 0.0 % [0/6] for primiparous vs multiparous women respectively)

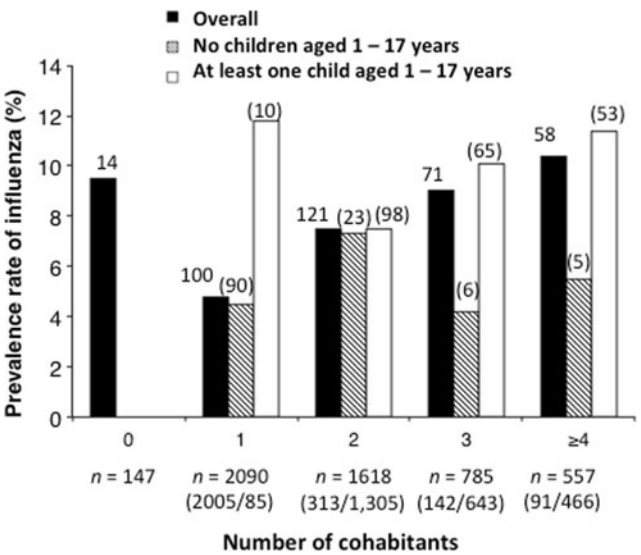

Fig. 3 Effects of number of cohabitants on the risk of influenza infection. The numbers of women with influenza are indicated at the tops of the bars. Compared to women with one cohabitant, women with 0, 2, 3, and ≥4 cohabitants had RR (95 % CI) of 1.99 (1.17 to 3.39), 1.56 (1.21 to 2.02), 1.89 (1.41 to 2.53), and 2.18 (1.60 to 2.96) for contracting influenza respectively. Women with cohabitant(s) were divided into two groups according to the presence or absence of at least one child aged 1–17 years. The presence of a child aged 1–17 years consistently increased the risk of influenza in pregnancy at all family sizes

risk of influenza infection in pregnancy increased with increasing number of cohabitants among women with at least one cohabitant, while pregnant women living alone (no cohabitant) were at higher risk of influenza comparable to that of pregnant women with ≥3 cohabitants in this study. Women with at least one child aged 1–17 years had a consistently higher risk of influenza at any family size. In addition, this study demonstrated that pregnant homemakers were at lower risk of influenza compared to women working outside the home. Taken together, these results suggested that pregnant women with a greater chance of encountering individuals possibly carrying influenza virus have higher risk of influenza. It was speculated that pregnant women living alone may have had more opportunities to go out compared to those with one cohabitant, and that nursery- and school-aged children were responsible for bringing influenza viruses into most families of multiparous pregnant women.

Our previous study conducted during the 2013–2014 influenza season indicated a seasonal influenza vaccine coverage rate among pregnant Japanese women of approximately 50 % [7], which was similar to those during and after the pandemic (H1N1) 2009 in the USA [8, 9], and was nearly equivalent between primiparous and multiparous women (50 and 53 % respectively [7], consistent with the results of 49 and 53 % respectively, in this study). However, multiparous women had a significantly higher rate of contracting influenza than primiparous women, regardless of vaccination status (5.6 % vs 2.2 % for vaccinated women and 9.7 % vs 3.5 % for unvaccinated women) [7]. This information was considered important and useful to aid national policy makers and health programme planners in making decisions about target groups for vaccination if this phenomenon would be reproducible. This study confirmed the reproducibility of the higher risk of influenza in multiparous than primiparous women; multiparous women had a higher risk of influenza regardless of vaccination status (RR of 1.80 [1.36–2.38] for vaccinated women and 2.18 [1.60–2.97] for unvaccinated women) compared to primiparous women.

We hypothesised that multiparous pregnant women have a greater number of cohabitants than primiparous women, and therefore the chance of influenza viruses being brought into the home is greater in families of multiparous than primiparous pregnant women. This hypothesis was verified in this study; indeed, family size was greater for multiparous than for primiparous women, and the risk of influenza among pregnant women increased with increasing number of cohabitants. Thus, the higher risk of influenza in multiparous than primiparous women could be explained by the greater numbers of cohabitants in families of multiparous women. In addition, it was suggested that nursery- and school-aged children could be responsible for bringing influenza viruses into most families of multiparous pregnant women.

Low vaccine effectiveness can occur as a result of a mismatch between vaccine strains and circulating strains [10], and was a concern in the 2014–2015 Northern hemisphere influenza season [11–13]. Indeed, vaccines used in Japan in the 2014–2015 influenza season were ineffective in reducing the number of pregnant women with influenza, with influenza prevalence rates of 7.3 % vs 6.7 % for those with and without vaccination, respectively, in this study.

It was difficult to verify whether respondents answered questions correctly due to the nature of this questionnaire study. However, the prevalence rate of influenza among unvaccinated pregnant women, 6.7 % (169/2526) in this study, was consistent with the corresponding rate of 6.3 % in the 2013–2014 influenza season [7]. Of women in this study who reported having contracted influenza, 84% (306/364) specified the types of influenza and 77 % (282/364) took antiviral agents. These figures were also consistent with those in the 2013–2014 influenza season (83 % for influenza type specification and 83 % for use of antiviral agents) [7]. In addition, low vaccine coverage in younger pregnant women was also a reproducible phenomenon; 28 % (95/336) for women aged <25 years in this study and 31 % in the 2013–2014 influenza season [7]. The use of influenza rapid diagnostic tests capable of differentiating between influenza A and influenza B is a common clinical practice in febrile patients in Japan, and the use of anti-influenza drugs for treatment of influenza is widely accepted in pregnant Japanese women [14].

In conclusion, this study demonstrated that multiparous women were at increased risk of influenza infection. The risk of influenza in pregnancy increased with increasing number of cohabitants. It was suggested that children aged 1–17 years could be responsible for bringing viruses into the homes of most families of multiparous pregnant women. Vaccine coverage was low in younger pregnant Japanese women. This information will be useful to aid national policy makers and health programme planners in making decisions about target groups for vaccination and intensified campaigns. Although mismatch between vaccine strains and circulating strains resulted in low vaccine effectiveness in the 2014–2015 influenza season in the Northern hemisphere [10–13], including Japan, maternal influenza immunization is a highly cost-effective intervention to reduce disease rates and severity in seasonal influenza epidemics as well as occasional pandemics [15]. Continued efforts are required to avoid the mismatch between vaccine strains and circulating strains and to encourage pregnant women to receive influenza vaccination.

Compliance with ethical standards

Disclosure All authors declare that they have no financial relationships with biotechnology manufacturers, pharmaceutical companies, or other commercial entities with an interest in the subject matter or materials discussed in this manuscript.

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