humans, indicating that neonates are exposed to PFCs via the placental passage (Inoue et al. 2004). Exposure of pregnant women and infants to PFCs had raised concerns, and they may have permanent neurodevelopmental toxic effects in humans.

However, the effects of prenatal PFCs exposure on neurodevelopment of next generation, especially in early infancy is not well understood. In the present analyses, we explored the relationship of prenatal PFOS/PFOA exposure with Mental and Psychomotor Developmental Indices (MDI, PDI) at 6 and 18 months of age assessed by Bayley Scales of Infant Development, 2nd edition (BSID II).

B. 研究方法

This study was a part of the Hokkaido Study on Environment and Children's Health performed between July 2002 and October 2005, and the details have been previously described (Kishi et al. 2011 and 2013). Of 1,796 approached Japanese pregnant women, 514 (28.6%) pregnant women agreed to participate in this study. A self-administered questionnaire survey was completed after the second trimester containing information related to smoking, economic status, educational levels, and alcohol and caffeine intake during pregnancy. Medical information including maternal age, maternal body mass index (BMI) before pregnancy, parity, gestational age, type of delivery, infant sex, and birth size were obtained from participant medical records.

A 40-mL blood sample was taken from the maternal peripheral vein after the second trimester of pregnancy, and PFOS and PFOA levels in maternal serum were measured by column-switching liquid chromatography -tandem mass spectrometry (LC/MS/MS). The PFOS values of all samples were detected, and for samples with PFOA levels below the detection limit (0.50 ng/mL), we used a value of half the detection limit (0.25 ng/mL). BSIDII were used to assess MDI and PDI in infants at 6 and 18 months of age. The environmental conditions of the subjects were assessed by using the questionnaire of home environment devised by Anme et al. (1997).

For the analysis of associations between maternal PFCs and BSID II, the following subjects were excluded: women with pregnancy-induced hypertension (n=11), women with diabetes mellitus (n=1), mother-infant pairs with fetal heart failure (n=1), and twins (n=7). After the exclusion of these subjects, 428 mother–infant pairs had available PFOS and PFOA concentrations. In addition, eligibility criteria for analysis of subjects were: babies born at term (37–42 weeks' gestation), Apgar score of > 7 at 1 min, infants without congenital anomalies or diseases, and BSID-II completed.

We analyzed correlations between PFC concentrations and the characteristics of the mothers and infants (also BSID II scores and characteristics) using the Spearman correlation test, the Mann–Whitney *U*-test, and the Kruskal–Wallis test. We performed multiple-regression analysis to examine the association between BSID-II scores and the levels of PFCs in maternal blood. The levels of PFCs in maternal blood were $\log_{10}$ transformed, and the analysis was adjusted for maternal age (year), parity (0/ ≥ 1), maternal educational levels (categorical), alcohol consumption and smoking during pregnancy (yes/no), caffeine intake during pregnancy (milligrams per day), blood sampling period (before and after delivery),

breast feeding (less or more than 3 months), Index of Child Care Environment, and total dioxin levels (TEQ, WHO 2005). We performed all of the statistical analyses using JMP pro 10 (SAS Institute Inc., NC, USA). Results were considered significant if $p < 0.05$.

（倫理面への配慮）

This study was conducted with the written informed consent of all participants and the study protocol was approved by the institutional ethical board for epidemiological studies at the Graduate School of Medicine and Center for Environmental and Health Sciences, Hokkaido University.

B. 研究結果

The basic characteristics of the study population are presented in Table 1. The mean scores were 90.5 (SD = 5.7) for MDI and 90.2 (SD = 10.3) for PDI at 6 months of age. Whereas, the mean scores for MDI and PDI were 84.2 (SD = 12.0) and 86.4 (SD = 10.9) at 18 months of age. The mean values of maternal PFOS and PFOA levels were 6.2 ng/mL and 1.3 ng/mL, respectively. In crude and adjusted model, PFOS and PFOA did not show significant association with MDI nor PDI at 6 months of age (n=174). After sex stratification, we found a negative significant association between prenatal PFOA and MDI score (but not PDI) only in female infants (β= -0.317; CI, -12.5 to -0.95; *p*-value= 0.023) in adjusted model. We did not find any significant association between PFOS levels with BSID II scores before and after sex stratification in adjusted model. Regarding the association between PFCs and neurodevelopment at 18 months (n=134), although there was a negative association between PFOA and MDI (β= -0.155; CI, -16.5 to 3.8; *p*-value= 0.219) and PDI (β= -0.135; CI, -14.2 to 4.3; *p*-value= 0.295) in adjusted model, but it was not significant. PFOS did not show any significant association with neurodevelopmental outcomes at 18 months of age. Sex stratification was applied to find sex differences of PFCs exposure on neurodevelopment but we did not find any significant association in either sexes at 18 months of age.

D. 考察

To our knowledge, this study is one of few epidemiological reports regarding the association between PFC levels and neurodevelopment, and the first study to examine correlation of PFCs and Bayley's test. We did observe no association between low prenatal PFOS exposure and BSID II scores at 6 and 18 months of age. We found negative correlation between prenatal PFOA exposure and MDI at 6 months of age only among female infants. We observed an inverse association between PFOA and MDI/PDI at 18 months of age but p-value was not significant. It may be due to small sample size in our study at 18 months of age (n=134).

There are few reports regarding the association of PFCs and neuro-development in infants and children. Fei et al. (2008) reported no convincing associations between prenatal PFCs (PFOS and PFOA) and developmental milestones at 6 and 18 months of age reported by mothers. In this report, PFOS and PFOA levels were higher than those in our study with a large sample size (6-months, n=1336; 18-months, n=1,255). In another recent study, there was no association between prenatal PFCs exposure and behavioral/affective disorders

such as ADHD in offspring (n=965) at 20 years of age (Strom et al. 2014). In contrast to our result, a Taiwanese report indicated PFOS but not PFOA levels in cord blood plasma were adversely associated with neurodevelopment among 2-year children using a national questionnaire (Chen et al. 2013). In our study, exposure levels of PFCs were lower than all of mentioned studies. Comparison of above results with our results is difficult due to difference of exposure levels, biomedia, societies, age of children and applied battery test. Our results suggest negative association between in utero PFOA exposure and neurodevelopment in early infancy especially among female infants.

E. 結論

Our result suggest in utero PFOA exposure is associated with lower neurodevelopmental indexes in early infancy especially among girls. Previously, our group reported time trends of 11 types of PFCs between 2003 and 2011 in plasma samples of pregnant women in Hokkaido (Okada et al. 2013). The results indicate that PFOS and PFOA concentrations declined, whereas long-chain PFCs (including PFNA and PFDA) levels increased. In future studies, assessment of the effects of newly emergent PFCs (such as PFNA and PFDA) on neurodevelopment of infants and children with bigger sample size and different battery tests is necessary.

F. 研究発表

1. 論文発表

In preparation

2. 学会発表

Houman Goudarzi, Tamiko Ikeno, Sachiko Kobayashi, Atsuko Araki, Chihiro Miyashita, Seiko Sasaki, Sonomi Nakajima, Hiroyuki Nakazawa, Reiko Kishi: Exposure to perfluoroalkyl chemicals and neurodevelopment at 6 months of age. International Society for Environmental Epidemiology (August 24-28, Seattle, USA, poster presentation)

G. 知的財産権の出願・登録状況（予定を含む。）

1.特許取得

なし

2.実用新案登録

なし

3.その他

なし

参考文献

1) Olsen GW, Burris JM, Ehresman DJ, Froehlich JW, Seacat AM, Butenhoff JL, et al. 2007. Half-life of serum elimination of perfluorooctanesulfonate perfluoro-hexanesulfonate, and perfluorooctanoate in retired fluorochemical production workers. Environ Health Perspect. 115:1298-305.

2) Lau C, Thibodeaux JR, Hanson RG, Rogers JM, Grey BE, Stanton ME,et al. 2003. Exposure to perfluorooctane sulfonate during pregnancy in rat and mouse. II: postnatal evaluation. Toxicol Sci.74:382-92.

3) Butenhoff JL, Ehresman DJ, Chang SC, Parker GA, Stump DG. 2009. Gestational and lactational exposure to potassium perfluorooctanesulfonate (K+PFOS) in rats: developmental neurotoxicity. Reprod Toxicol. 27:319-30.

4) Inoue K, Okada F, Ito R, Kato S, Sasaki S, Nakajima S, et al. 2004. Perfluorooctane sulfonate (PFOS) and related perfluorinated compounds in human maternal and cord blood samples: assessment of PFOS exposure in a susceptible population during

pregnancy. Environ Health Perspect. 112:1204-7.
5) Kishi R, Sasaki S, Yoshioka E, Yuasa M, Sata F, Saijo Y, et al. 2011. Cohort profile: the Hokkaido study on environment and children's health in Japan. Int J Epidemiol. 40:611-8.
6) Kishi R, Kobayashi S, Ikeno T, Araki A, Miyashita C, Itoh S, et al. 2013. Ten years of progress in the Hokkaido birth cohort study on environment and children's health: cohort profile--updated 2013. Environ Health Prev Med. 18:429-50.
7) Anme T, Shimada C, Katayama H. 1997. Evaluation of environmental stimulation for 18 months and the related factors. Nihon Koshu Eisei Zasshi. 44:346-52.
8) Fei C, McLaughlin JK, Lipworth L, Olsen J. 2008. Prenatal exposure to perfluorooctanoate (PFOA) and perfluorooctanesulfonate (PFOS) and maternally reported developmental milestones in infancy. Environ Health Perspect. 116:1391-5.
9) Strøm M, Hansen S, Olsen SF, Haug LS, Rantakokko P, Kiviranta H. et al. 2014. Persistent organic pollutants measured in maternal serum and offspring neurodevelopmental outcomes--a prospective study with long-term follow-up. Environ Int. 68:41-8.
10) Chen MH, Ha EH, Liao HF, Jeng SF, Su YN, Wen TW. et al. 2013. Perfluorinated compound levels in cord blood and neurodevelopment at 2 years of age. Epidemiology. 24:800-8.
11) Okada E, Kashino I, Matsuura H, Sasaki S, Miyashita C, Yamamoto J. et al. 2013. Temporal trends of perfluoroalkyl acids in plasma samples of pregnant women in Hokkaido, Japan, 2003-2011. Environ Int. 60:89-96.

Table 1. Characteristics of mothers and infants

Characteristics	At 6 months No. (%)	At 18 month No. (%)
Maternal characteristics		
Age (years) [a]	30.8±4.6	31.1±4.4
Prepregnancy BMI (kg/m2) [a]	21.2±2.9	21.2±2.9
Parity (times)		
0	83 (48.0)	65 (48.9)
≥1	90 (52.0)	68 (51.1)
Smoking during pregnancy		
Yes	53 (30.5)	39 (29.1)
No	121 (69.5)	95 (70.9)
Alcohol intake during pregnancy		
Yes	51 (29.3)	40 (29..9)
No	123 (70.0)	94 (70.1)
Blood sampling period		
during pregnancy	132 (75.9)	107 (79.8)
after delivery	42 (24.1)	27 (20.1)
Child characteristics		
Sex		
Male	84 (48.3)	67 (50.0)
Female	90 (51.7)	67 (50.0)
Gestational age (days) [a]	276.4±8.3	276.4±8.5
Birth weight (g) [a]	3112.5±357.9	3098.6±323.3
Birth length (cm) [a]	48.2±1.7	48.2±1.6
Breast-feeding (month)		
<3	72 (41.4)	51 (38.0)
≥3	102 (58.6)	83 (62.0)
BSID II mental index score (MDI) [a]	90.5±5.7	84.2±12.0
BSID II psychomotor index score (PDI) [a]	90.2±10.3	86.4±10.9
Index of Child Care Environment [b]	22.2±3.9	28.2±3.5

[a]mean±SD. [b]perfect score is 30 and 38 points in 6 months and 18 months, respectively.

Table 2.

Concentration of PFOS and PFOA (ng/mL) in maternal blood (n=174) whose infants had Bayley test score

	Mean (SD)	Minimum	Percentile			Maximum
			25th	50th	75th	
PFOS	6.2 (2.7)	1.6	4.4	5.7	7.4	16.2
PFOA [a]	1.3 (0.8)	not detected	0.8	1.2	1.7	4.3

Concentration of PFOS and PFOA (ng/mL) in maternal blood (n=134) whose infants had Bayley test score

	Mean (SD)	Minimum	Percentile			Maximum
			25th	50th	75th	
PFOS	6.2 (2.6)	1.70	4.4	5.8	7.4	16.2
PFOA [a]	1.3 (0.8)	not detected	0.8	1.2	1.8	4.3

[a] For subjects with a level below the detection limit, a value equal to half the detection limit were used.

Table 3. BSID II MDI and PDI development scores at 6 months of age for infants in relation to the levels of PFCs in maternal blood (n=174).

	MDI			PDI		
	beta	(95% CI)	p-value	beta	(95% CI)	p-value
PFOS						
crude	0.037	(-3.26 to 5.43)	0.624	-0.016	(-8.62 to 6.96)	0.833
Adjusteda	0.036	(-4.02 to 6.08)	0.688	-0.004	(-0.391 to 0.371)	0.959
PFOA						
crude	0.003	(-2.89 to 3.03)	0.961	-0.030	(-6.38 to 4.21)	0.687
Adjusted a	-0.033	(-4.16 to 2.88)	0.721	0.011	(-5.31 to 6.08)	0.893

adjusted for gestational age, parity, maternal age, smoking and alcohol intake during pregnancy, caffeine during pregnancy, maternal education level, blood sampling period, breast feeding, score, Index of Child care Environment, and total dioxin levels (TEQ, WHO 2005).

Table 4. BSID II MDI and PDI development scores at 6 months of age for infants in relation to the levels of PFCs in maternal blood by sex stratification.

Boys (n=84)	MDI			PDI		
	beta	(95% CI)	p-value	beta	(95% CI)	p-value
PFOS						
crude	-0.080	(-8.58 to 3.96)	0.466	-0.041	(-12.21 to 8.32)	0.707
adjusted[a]	-0.064	(-9.84 to 6.26)	0.659	0.178	(-3.62 to 19.01)	0.179
PFOA						
crude	0.087	(-2.38 to 5.52)	0.431	0.009	(-6.20 to 6.74)	0.934
adjusted[a]	0.139	(-2.09 to 7.72)	0.368	0.063	(-5.87 to 9.33)	0.650
Girls (n=90)	MDI			PDI		
	beta	(95% CI)	p-value	beta	(95% CI)	p-value
PFOS						
crude	0.136	(-2.11 to 10.03)	0.198	0.002	(-11.63 to 11.90)	0.981
adjusted[a]	0.077	(-5.08 to 9.55)	0.545	0.029	(-11.27 to 14.48)	0.804
PFOA						
crude	-0.094	(-6.56 to 2.48)	0.373	-0.055	(-10.97 to 6.43)	0.605
adjusted[a]	-0.317	(-12.5 to -0.95)	**0.023**	0.096	(-6.59 to 14.46)	0.458

Adjusted for gestational age, parity, maternal age, smoking and alcohol intake during pregnancy, caffeine during pregnancy, maternal education level, blood sampling period, breast feeding, Index of Child Care Environment, and total dioxin levels (TEQ, WHO 2005).

Table 5. BSID II MDI and PDI development scores at 18 months of age for infants in relation to the levels of PFCs in maternal blood (n=134).

	MDI			PDI		
	beta	(95% CI)	p-value	beta	(95% CI)	p-value
PFOS						
crude	0.082	(-5.77 to 16.5)	0.342	-0.033	(-12.08 to 8.15)	0.701
adjusted[a]	-0.002	(-17.8 to 17.4)	0.985	-0.030	(-18.0 to 14.2)	0.815
PFOA						
crude	-0.090	(-10.9 to 3.3)	0.298	-0.065	(-8.9 to 4.0)	0.455
adjusted[a]	-0.155	(-16.5 to 3.8)	0.219	-0.135	(-14.2 to 4.3)	0.295

[a] adjusted for gestational age, parity, maternal age, smoking during pregnancy, alcohol during pregnancy, caffeine during pregnancy, maternal education, blood sampling period, breast feeding, caffeine during pregnancy, maternal education, blood sampling period, breast feeding, Index of Child Care Environment, and total dioxin levels (TEQ, WHO 2005).

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分担研究報告書

Effects of Prenatal Exposure to Perfluoroalkyl Acids on Cord Blood *IGF2/H19* Methylation and Associations with Birth Size

研究代表者 岸 玲子 北海道大学環境健康科学研究教育センター 特任教授
研究分担者 荒木 敦子 北海道大学環境健康科学研究教育センター 准教授
研究分担者 佐々木 成子 北海道大学大学院医学研究科社会医学講座公衆衛生学分野 助教
研究分担者 石塚 真由美 北海道大学大学院獣医学研究科環境獣医科学講座毒性学教室 教授

研究要旨

Background: Prenatal exposure to perfluoroalkyl acids (PFAAs) can influence fetal growth and postnatal obesity. However, only one previous study has examined the epigenetic effects of PFAAs *in utero*. Specifically, the effects on gene-specific methylation, including the fetal growth factor insulin-like growth factor 2 (IGF2), and the consequences to the offspring are unknown.

Objectives: To evaluate the effects of prenatal PFAA exposure on infants' *IGF2/H19* and *LINE1* DNA methylations and their relations to birth size.

Methods: Two hundred thirty-five mother-child dyads from the Hokkaido Study on Environment and Children's Health were included in this study. Perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) levels in the maternal sera were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The *IGF2* differentially methylated region (DMR), *H19* DMR and *LINE1* methylation were quantified in cord blood by pyrosequencing. Multiple linear regressions were performed.

Results: The median concentrations of PFOS and PFOA were 5.0 and 1.4 ng/mL, respectively. In the fully adjusted model, the *IGF2* methylation fractions significantly decreased with a log10-increase in PFOA concentration [β = -1.53, 95% confidence interval (CI): -2.93 to -0.12]. We observed a positive correlation between *IGF2* methylation and the ponderal index (kg/m^3) at birth (β = 0.18, 95% CI: 0.09 to 0.27).

Conclusion: Our results suggest that prenatal PFOA exposure results in *IGF2* hypomethylation in the offspring, which explains some of the decrease in the ponderal index at birth. Further investigations are necessary to elucidate the effects of *IGF2* hypomethylation on the offspring's postnatal growth and obesity.

研究協力者
小林 祥子、安住 薫、Houman Goudarzi、
小林 澄貴
（北海道大学環境健康科学研究教育センター）
中澤 裕之
（星薬科大学薬品分析化学教室）

A．研究目的

Perfluoroalkyl acids (PFAAs) are currently widely used synthetic compounds for consumer products and industrial applications and are dispersed all over the environment (Lau et al. 2007). PFAAs have long half-lives of 5.4 years for perfluorooctane sulfonate (PFOS) and 3.8 years for perfluorooctanoic acid (PFOA) in humans (Olsen et al. 2007) and it was reported that PFOS and PFOA are both able to pass through the placental barrier and might exert negative effects on fetuses (Midasch et al. 2007). Previous animal studies have indicated that PFAAs are developmentally toxic and causes neonate mortality and reduced body weight (Kudo and Kawashima 2003). We previously reported that prenatal exposure to PFOS significantly reduces the birth weight of female infants (Washino et al. 2009) and this finding has also been supported by other studies (Olsen et al. 2009). Additionally, in a recent Danish birth cohort study, prenatal PFOA exposure was found to be associated with overweight and obese statuses among 20-year-old females and changes in their obese biomarkers, which suggests that modifications due to PFOA exposure in utero persist until adulthood (Halldorsson et al. 2012). Recent researches indicated that environmental chemicals in utero may perturb epigenetic machinery and result in persistent phenotypic changes in whole life (Baccarelli and Bollati 2009). We hypothesized prenatal PFAA exposure modifies the DNA methylation patterns of infants and results in reduced birth size.

DNA methylation is an epigenetic process that shapes the cell's characteristics over its entire life and thus has been postulated to be a mediator of the effects of the intrauterine environment on postnatal phenotypes (Drong et al. 2012). DNA methylation occurs via the addition of a methyl group to a cytosine at cytosine–guanine dinucleotides (CpGs) and acts like a "switch" for gene expression. DNA methylation plays fundamental roles during embryonic development and cell differentiation by establishing tissue–specific DNA methylation patterns and subsequent stable gene expression (Hackett and Surani 2013). Additionally, these methylation patterns can be heritable through cell division and are influenced by the external environment (Fraga et al. 2005). Previous epidemiological studies have suggested that prenatal exposure to maternal smoking and environmental chemicals, such as persistent organic pollutants (POPs), bisphenol A and heavy metals, modifies the epigenome of whom exposed (Baccarelli and Bollati 2009).

PFOA has also been reported to have epigenetic effects on cord blood global DNA methylation; however, the study that reported this finding was cross–sectional and had a small sample size (N=30) (Guerrero-Preston et al. 2010).

One of the most studied regions in epigenetics is the *insulin–like growth factor 2 (IGF2)/H19* locus. IGF2 is a growth hormone that is essential for fetal growth and is expressed in throughout fetal development. Secreted IGF2 exerts mitogenic and metabolic effects on all tissues, and polymorphisms of this gene are associated with fetal growth (Kaku et al. 2007). Additionally, methylations of *IGF2/H19* in cord blood and placental tissues have been found to be associated with fetal growth in human epidemiological studies. In the Generation R study, children born small-for-gestational age (SGA) were found to exhibit lower *IGF2* methylation levels in cord blood; interestingly, lower *IGF2* methylation was also associated with greater weight gain in the first three months after birth (Bouwland-Both et al. 2013). Other previous study demonstrated that *IGF2/H19* placental methylation and genetic polymorphisms together account for 31% of birth weight variance (St-Pierre et al. 2012), which is suggestive of a major role of the *IGF2* in fetal growth. Moreover, emerging evidence has indicated the role of *IGF2* gene in metabolic risks in humans. Several reports have shown that polymorphisms of *IGF2* are associated with weight and the obese phenotypes (Cianfarani 2012). Additionally, a recent report suggested that *IGF2/H19* methylation at birth is linked to the development of overweight or obesity in early childhood (Perkins et al. 2012).

The expression of this imprinted gene is controlled by DNA methylation at the *IGF2* and *H19* differentially methylated regions (DMRs), which are reciprocally methylated and expressed between the maternal and paternal alleles (Gabory et al. 2010). In addition to their functional importance, these regions have been suggested to be epigenetically labile to environmental perturbations, especially in early life, and have been studied as indicators of epigenetic disruption (Hoyo et al. 2009). Previous epidemiological studies have demonstrated that perturbation in the intrauterine nutritional environment can decrease *IGF2* methylation in the offspring. Heijmans et al. reported that 60 year–old individuals who had experienced severe famine in utero during the Dutch Huger Winter, exhibited 5.2% less IGF2 methylation in their peripheral blood than their unexposed, sex–matched siblings (Heijmans et al. 2008). Moreover, the children of mothers without periconceptual folic acid supplementation exhibit lower *IGF2* methylation in their cord blood (Haggarty et al. 2013) and peripheral

blood during infancy (Steegers-Theunissen et al. 2009). Other marker of epigenetic disruptions include repetitive sequences, such as the *Long interspersed element 1 (LINE1)*, which is a retrotransposon sequence that is globally distributed and composes approximately 17% of the human genome, and has thus been used as a surrogate for a global methylation (Cortessis et al. 2012).

In this birth cohort study, we prospectively evaluated the effects of prenatal PFOS and PFOA exposures on gene–specific DNA methylation at *IGF2/H19* and global methylation of *LINE1* and the associations of these effects on fetal growth.

B．研究方法

2.1. Study population. The study participants were enrolled as a part of the Hokkaido Study on Environment and Children's Health, which is an ongoing birth cohort study. Details about the study design have previously been described (Kishi et al. 2011; Kishi et al. 2013). Briefly, pregnant women at 23–35 weeks of gestation were recruited at the Toho Hospital in Sapporo, Japan between 2002 and 2005. Of the 1,796 eligible women, some were excluded because they were registrants of the Japanese cord blood bank (25%) or were planning to deliver at another hospital (3%). Ultimately, 514 expectant mothers agreed to participate in this cohort (29% participation rate). Among the 514 pregnant women who participated at the enrollment, 10 were excluded due to miscarriage or stillbirth (N = 2), relocation (N = 1), and voluntary withdrawal (N = 7) from the study before follow–up. From these mothers, 447 maternal blood samples and 267 cord blood samples were available for PFAA and DNA methylation analyses. Subsequently, 235 mother-infant dyads whose PFAA and DNA methylation data were available were included in this study. All 235 deliveries were singletons.

This study was conducted with the written informed consent of all subjects. The institutional ethical board for human gene and genome studies at Hokkaido University Center for Environmental and Health Science and Hokkaido University Graduate School of Medicine approved the study protocol.

2.2. Data collection. At enrollment, a self-administered questionnaire was utilized to obtain baseline information that included parental demographic characteristics, anthropometric measurements and lifestyle factors, such as maternal smoking and alcohol consumption (Washino et al. 2009). Information about pregnancy complications, gestational age, infant gender and birth size was obtained from the medical records.

2.3. Specimen collection. A 40-mL maternal blood samples were collected from 177 participants at between 24-41 weeks of gestational age (75.3%) and

from 58 participants within five days of delivery (24.7%). Cord blood samples were taken immediately at birth. All specimens were stored at -80°C until the time of analyses.

2.4. Exposure assessment. Detailed methods for the measurements of PFOS and PFOA have previously been described (Inoue et al. 2004; Nakata A 2009). Serum samples (0.1 mL) were mixed with 0.2 mL internal standard solution containing acetonitrile and centrifuged at 1450 × g for 10 min, and the supernatants were transferred to a polypropylene tube. An aliquot of the filtered sample solution was subjected to column-switching liquid chromatography-tandem mass spectrometry (LC–MS/MS). PFOS was detected in all of the samples, and the PFOA levels of 15 samples were below the detection limit (0.50 ng/mL). For PFOA concentrations of those samples, we used a value of half the detection limit (0.25 ng/mL).

2.5. Quantification of DNA methylation. Genomic DNA was extracted from umbilical cord blood using a Maxwell® 16 DNA Purification Kit (Promega, Madison, WI, USA). 500 ng of DNA were subjected to a bisulfite conversion using an Epitect Plus Bisulfite Kit (Qiagen, Venlo, Netherlands) to convert the unmethylated cytosines to uracils while leaving the methylated cytosines unchanged. Bisulfite pyrosequencing was performed using previously described methods and primers (Bollati et al. 2007; Murphy et al. 2012a). We evaluated two *IGF2/H19* regulatory regions, one involved the two CpG sites comprising the intragenic *IGF2* DMR0, and the other involved the four CpG sites within the *H19* DMR, which is within the *IGF2/H19* imprinting control region (ICR). For *LINE1*, three CpG sites in promoter region were quantified. Each locus was amplified using HotStarTaq DNA polymerase in PyroMark PCR Kits (Qiagen). Biotin-labeled single-stranded amplicons were bound to the Streptavidin Sepharose HP (Amersham Biosciences, Uppsala, Sweden) and purified using the Pyromark Q24 Work Station (Qiagen). Pyrosequencing was performed using a Pyromark Q24 system, and the data were analyzed using Pyro Q-CpG Software (Qiagen). The PCR primers, conditions and sequencing primers are described in Table S1. All of the samples were analyzed in duplicate. For the subsequent analyses, the average methylation level of each CpG site was used.

2.6. Data Analyses. The Spearman correlation test, Mann–Whitney U-test, and Kruskal–Wallis test were applied to determine whether the maternal and offspring characteristics were associated with PFAA exposure. Multiple linear regression analyses were performed to determine the associations between the maternal PFAA concentrations and the infants'

methylation levels at each locus. Due to skewed distributions, we transformed the concentrations of PFOS and PFOA concentrations into log10 scales. The maternal blood sampling periods for the PFAA measurements were categorized into four groups: < seven months, eight months ± four weeks, > nine months of pregnancy, and after delivery. Additionally, the samples were divided into quartiles according to PFOS and PFOA concentrations, and tests for a linear trend were performed by linear contrast. The adjusted least square means of the methylation levels in each quartile were compared with the Hsu-Dunnet method to accommodate for multiple comparisons. Multiple linear regressions were applied to estimate the effects on the infants' birth sizes. The infant ponderal index, an indicator of relative soft tissue mass to bone structure, was calculated as the ratio of birth weight (kg) to cubed birth length (m^3) (Miller and Hassanein 1971). One subject was excluded from the analysis of birth length and ponderal index because the birth length of this infant was more than four standard deviations (SDs) from the mean despite having a gestational age and birth weight within the normal ranges. The DNA methylation model was adjusted for maternal age, education, the infant's sex, maternal smoking during pregnancy and the blood sampling period. The birth size model was adjusted for gestational age, maternal age, pre-pregnancy body mass index (BMI), parity, maternal education, maternal smoking during pregnancy and the infant's sex. All statistical analyses were conducted using JMP pro 11 (SAS Institute Inc., NC, USA). P-values below 0.05 were considered statistically significant.

（倫理面への配慮）

本研究は，北海道大学環境健康科学研究教育センターおよび北海道大学大学院医学研究科・医の倫理委員会の承認を得た。本研究によって得られた個人名及び個人データの漏洩については，データの管理保管に適切な保管場所を確保するなどの方法により行うとともに，研究者の道義的責任に基づいて個人データをいかなる形でも本研究の研究者以外の外部の者に触れられないように厳重に保管し，取り扱った。

C．研究結果

Among the 235 mother-infant dyads who were included in the study, the mean methylation levels (±SD) for the *IGF2* DMR0, *H19* DMR and *LINE1* were 49.1% (±3.3), 52.1% (±2.0) and 75.7% (±1.2), respectively. The geometric means (GM) (25th to 75th percentiles) of the PFOS and PFOA levels in the maternal blood were 4.8 ng/mL (3.3 to 6.8) and 1.2 ng/mL (0.9 to 1.9), respectively (Table 1). The maternal and infant characteristics and their relations to the PFOS and PFOA concentrations are described in Table 2.

Table 3 shows the results of the multiple linear regression analyses of the effects of the PFAAs on DNA

methylation in the cord blood. After adjusting for potential confounders, our results demonstrated a significant decrease in *IGF2* methylation fraction with a log10-unit increase in PFOA (β = -1.53, 95% CI: -2.93 to -0.12). Although not statistically significant, the PFOS level exhibited a trend similar to that of PFOA (β = -0.90, 95% CI: -2.79 to 0.98). The *H19* and *LINE1* methylations did not exhibit any significant association with either PFOS or PFOA concentrations.

Next, we analyzed the effects of PFOS and PFOA stratified by infant gender (Table 4). We did not find significant associations among either the male or female infants. However, after adjusting for potential confounders, a stronger association between PFOS and *IGF2* methylation was observed among the female infants (β = -2.30, 95% CI: -4.92 to 0.31) compared to the male infants.

Figure 1 shows the changes in the adjusted least square means in *IGF2* methylation across the PFOS and PFOA quartiles. After adjusting for potential confounders, we found that, although only marginally significant, *IGF2* methylation was decreased by 1.31% in the fourth PFOA quartile (> 1.9 ng/mL) compared to the first quartile (≤ 0.9 ng/mL) (β = -1.31, 95% CI: -2.65 to 0.03, p = 0.06). The results also revealed a decrease from the second to fourth quartiles (p for trend = 0.01 in all quartiles). The PFOS quartiles did not exhibit any significant relation to *IGF2* methylation (p for trend = 0.29; Table S3).

We analyzed whether DNA methylation at *IGF2* was associated with fetal growth (Table 5). After adjusting for gestational age (Model 1) and other potential confounders (Model 2), *IGF2* methylation exhibited a significant correlation with the ponderal index (β = 0.18, 95% CI: 0.09 to 0.27). *H19* methylation was not significantly related to these fetal growth indices (data not shown).

D．考察

The prenatal period is a critical time for a fetus to create tissue-specific DNA methylation patterns to develop a functional human body, and perturbation of these canonical processes by external factors such as environmental chemicals might result in persistent epigenetic and phenotypic changes (Barnes and Ozanne 2011). In this study, even at our daily-life exposure levels, we found that prenatal PFOA exposure resulted in a decrease in DNA methylation at the *IGF2* locus in cord blood, which was associated with a lower ponderal index at birth. Interestingly, these effects were more pronounced in the gene-specific methylation than global methylation as measured at *LINE1.* To our knowledge, this is the first epidemiological report to evaluate the effects of prenatal PFAA exposure on gene-specific methylation.

Previous epidemiological studies have demonstrated that intrauterine nutritional perturbations, such as severe famine (Heijmans et al. 2008) and perinatal maternal folic acid deficiencies (Haggarty et al. 2013; Steegers-Theunissen et al. 2009), can decrease the offspring's *IGF2* methylation. According to previous animal and epidemiological studies, prenatal exposure to PFOA can result in poor growth in utero (Olsen et al. 2009). Our results indicated that prenatal PFOA exposure, which is another possible cause of fetal growth restriction, can elicit effects similar to those of malnutrition and folic acid deficiency and resulted in hypomethylation at the *IGF2* locus.

In the current study, we observed that *IGF2* methylation was positively associated with the ponderal index at birth (β = 0.18, 95% CI: 0.09 to 0.27). Based on our observations, it can be estimated that a ten-fold increase in PFOA concentration resulted in a 1.53% decrease in *IGF2* methylation, which accounted for a reduction in the ponderal index of 0.28 kg/m3. However, because our observations of the association of DNA methylation and ponderal index were cross-sectional, we were unable to determine whether lower ponderal indices resulted in *IGF2* hypomethylation or vice versa. Moreover, it is unknown whether the minor *IGF2* hypomethylation in the cord blood that we observed could represent functional consequences such as changes in *IGF2* protein levels.

The relationships of *IGF2* methylation with IGF2 protein levels have not yet been fully clarified. A previous study of placental tissues, which actually secrete IGF2, showed that placental *IGF2* DMR0 methylation is positively correlated with IGF2 protein concentration in the cord blood, which in turn is positively associated with fetal growth indices (St-Pierre et al. 2012). Whereas another cord blood study reported a negative correlation between cord blood *IGF2* DMR0 methylation and IGF2 protein circulation (Hoyo et al. 2012), which contradicts our observations. Mechanistically, DNA methylation at the *H19* DMR is thought to play a more crucial role in *IGF2* expression; *H19* DMR is located in the *IGF2/H19* ICR and determines the expression of the *H19* gene and the inhibition of *IGF2* expression (Gabory et al. 2010). However, our results showed that methylation at the *IGF2* DMR was associated with the ponderal index, and we do not have a rational explanation for this matter yet.

In contrast, recent epidemiological studies have suggested an association between methylations of imprinted genes and postnatal growth. It has been reported that one year-old infants within >85th percentile in weight for age have higher *H19* methylation levels in the cord blood (Perkins et al. 2012), and *IGF2/H19* ICR methylation in the

peripheral blood was found to be positively correlated with subcutaneous adiposity and skinfold thickness at 17 years of age in cross-sectional study (Huang et al. 2012). It is necessary to further follow up the growth of our population that was exposed to PFOA in utero and exhibited *IGF2* hypomethylation.

Although the exact modes of action of the effects of PFOA on DNA methylation remain to be elucidated, some potential manners in which prenatal PFOA exposure might affect DNA methylation are known. As exogenous ligands, PFAAs might actively change the DNA methylation of specific genes by binding to their target receptors. Previous in vitro reporter assays have demonstrated that PFOA and PFOS can activate the human *peroxisome proliferator - activated receptors (PPARs)* α and γ (Vanden Heuvel et al. 2006), which are important nuclear receptors for lipid homeostasis, adipogenesis and other physiological processes. A previous study suggested that PPAR γ can induce changes in histone modifications, which represent another epigenetic mechanism that is closely linked to DNA methylation status, and promote adipogenesis (Wakabayashi et al. 2009). These findings might explain the gene-specific methylation due to PFOA exposure that was observed. Moreover, it is possible that other environmental chemicals with the ability to activate PPARs also elicit effects similar to those of PFOA, and further evaluations are necessary.

In addition to transactivating PPARs, PFOA and PFOS can also exhibit weak estrogenic activity by directly interacting with estrogen receptors (ERs) (Kjeldsen and Bonefeld-Jorgensen 2013), which can lead to changes in *IGF2* methylation (Pathak et al. 2010). This estrogen interaction might explain the sex difference in methylation due to PFOS exposure observed in our study. However, this result was no statistically significant, and further studies with larger samples are needed to clarify the sex differences in the epigenetic effects of PFAAs.

In our study, we observed that only PFOA decreased *IGF2* methylation. A previous in vitro reporter assay indicated that PFOA is a stronger activator of PPARs than PFOS (Takacs and Abbott 2007). Moreover, it has been shown that PFOA has greater transplacental transfer efficiency (Beesoon et al. 2011). These findings might explain our observation that PFOA had a stronger effect on *IGF2* hypomethylation despite the lower exposure level to PFOA than PFOS.

Regarding global methylation, we did not observe a significant association between PFAA concentration and *LINE1* methylation. A previous cross-sectional study (N = 30) demonstrated that PFOA concentrations in cord blood are inversely correlated with the

offspring' s global DNA methylation as quantified with enzyme-linked immunosorbent assay (ELISA) (Guerrero-Preston et al. 2010), which is an immunochemical method that can be used to quantify total methylated cytosine to measure global methylation. In contrast, we measured the methylation of the retrotransposon sequence *LINE1* as a surrogate marker of global methylation. Choi et al. reported that *LINE1* methylation is not correlated with total cytosine methylation (Choi et al. 2009). These methodological differences might have caused the discordance of the two results. In contrast, two studies in adult populations indicated that PFOA concentrations are not significantly associated with *LINE1* methylations in peripheral blood or sperm cells (Leter et al. 2014; Watkins et al. 2014). Moreover, two in vitro studies reported no significant effects of PFOA on total cytosine methylation in human cells (Bastos Sales et al. 2013; Tian et al. 2012), and Tian et al. reported a significant increase in methylation at a gene promoter region. The results of these previous studies are concordant with our observations, which suggests that PFOA exposure exerts greater effects on the DNA methylation of specific genes rather than the global methylation.

Our study is a birth cohort study that has followed participants since the prenatal period and can thus minimize recall bias. However, we acknowledge that this study also has some limitations. First, we measured DNA methylation in cord blood samples, which might not entirely represent the methylation levels of other tissues. Comparisons of the methylation levels across tissues have demonstrated that the *H19* methylation levels are similar across tissues, but lower levels of *IGF2* methylation have been observed in the brain, kidneys and liver compared to other tissues including cord blood and the placenta (Murphy et al. 2012b). Secondly, our study did not have a sufficient sample size to ensure adequate statistical power; thus, it is possible that we were unable to detect all of the effects of PFAA on *IGF2/H19* and *LINE1* methylations. Third, population of this study was entirely from a single hospital in Sapporo city; however, compared to another of our birth cohort studies that covered 37 hospitals in Hokkaido prefecture, the characteristics of the participants were very similar (Kishi et al. 2011; Kishi et al. 2013), which suggests the hospital from which we recruited participants was a typical hospital without special deviations. Therefore, our results can be generalized to a larger context. Additionally, we only used the data from the participants who had cord blood samples for DNA methylation analyses, and this limited our study population to those with vaginal deliveries (99.6%). Another limitation is that the maternal

blood sampling period for the PFAA measurements differed between the subjects. However, PFOS and PFOA have relatively long half-life of 5.4 years and 3.8 years (Olsen et al. 2007), and we adjusted for the blood sampling period to minimize its influence on our results.

In the current study, we found that in utero PFOA exposure can decrease DNA methylation at the *IGF2* locus. In 2009, PFOS was classified as Annex B of the Stockholm Convention on POPs. Since that time, the use of PFOS and PFOA has been declining worldwide (Olsen et al. 2012). Similarly, our group reported that plasma PFOS and PFOA concentrations also decreased in Japan between 2003-2011, whereas the concentrations of perfluorononanoic acid (PFNA) and perfluorodecanoic acid (PFDA) have increased over the years (Okada et al. 2013). Therefore, it is necessary to evaluate the effects of these newly emerged PFAAs on the fetal epigenome. Additionally, it is plausible that PFOA and other PFAAs might exert their epigenetic effects on genomic regions other than *IGF2*. Further epigenome-wide association studies are necessary to fully elucidate the influence of prenatal PFAA exposure on the offspring' s epigenome and future risks.

E．結論

Our study indicated that prenatal exposure to PFOA decreased *IGF2* methylation in infants but did not significantly affect *H19* or *LINE1* methylation. Moreover, lower *IGF2* methylation levels were associated with lower ponderal indices at birth, which might explain the involvement of DNA methylation in fetal growth restriction that is caused by PFOA exposure. Further long-term observations are necessary to evaluate the health effects of prenatal PFOA exposure and the subsequent *IGF2* hypomethylation on health and disease of the offspring.

F．研究発表

1．論文発表

(1) Kobayashi S, Azumi K, Itoh S, Goudarzi H, Sasaki S, Ishizuka M et al. Effects of Prenatal Exposure to Perfluoroalkyl Acids on Cord Blood IGF2/H19 Methylation and Associations with Birth Size: The Hokkaido Study. Environ Int (submitted)

(2) 安住 薫, 小林 祥子, 岸 玲子; 環境化学物質曝露の次世代影響の解明におけるエピジェネティクス研究. 北海道公衆衛生学雑誌. 26(2):29-38, 2012.

2．学会発表

(1) Kobayashi S., Azumi K., Sasaki S., Ishizuka M., Nakazawa H., Okada E., Kobayashi S., Goudarzi H., Itoh S., Miyashita C., Ikeno T., Araki A., Kishi R.; The effects of perfluoroalkyl acids (PFAAs) exposure in utero on IGF2/H19 DNA methylation in cord blood. 26th Annual International Society for