Fetal Environment and Glycosylation Status in Neonatal Cord Blood

A Comprehensive Mass Spectrometry-based Glycosylation Analysis

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Abstract: Fetal environment is known to be a major predictive factor of type 2 diabetes and cardiovascular disease. However, associations of fetal environment and cord blood glycoforms are uncertain. In this study, we aimed to determine whether glycosylation status in neonatal cord blood is associated with perinatal outcomes reflecting a poor fetal environment.

Thirty-six low birth weight (LBW) infants and 120 normal birth weight infants were recruited from a longitudinal birth cohort. We conducted a comprehensive cord blood N-glycan analysis using matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry. Associations of N-glycans with perinatal outcomes, including LBW, small for gestational age, and levels of cord blood leptin and adiponectin, were evaluated using logistic or multiple regression. We also prospectively explored correlations between N-glycans and 6 or 18-month rapid weight gain (>0.67 SD score).

A total of 35 N-glycans were detected (m/z value 1362.481–3865.407). Of these, abundance levels of G3414 (m/z value 3414.238) were inversely correlated with LBW and small for gestational age. Abundance levels of G1915 (m/z value 1914.698), G2744 (m/z value 2743.994), G3049 (m/z value 3049.105), and G3719 (m/z value 3719.349) were inversely related to LBW. The total N-glycan abundance levels were strongly positively correlated with levels of leptin and adiponectin in cord blood. In a prospective exploratory analysis, the 5 LBW-related N-glycans (G1915, G2744, G3049, G3414, and G3719) were all inversely associated with 6 or 18-month rapid weight gain. These N-glycans are structurally categorized into 2 different categories: fucosylated bi or tri-antennary N-glycans; and tri or tetra-antennary N-glycans without fucosylation.

In conclusion, mass spectrometry-based cord blood glycosylation analysis shows that 5 types of N-glycans are potential predictors of a poor fetal environment.

INTRODUCTION

The fetal environment is an important area of research because it is associated with various diseases including type 2 diabetes and cardiovascular disease in later life.1,2 Fetal malnutrition in utero, which prevents appropriate fetal growth, is thought to provoke a thrifty phenotype in premature infants. This phenotype is assumed to predispose these infants to subsequent type 2 diabetes and cardiovascular disease. Low birth weight (LBW) and small for gestational age (SGA) are known to be manifestations of in utero stress. Epidemiological studies have shown that LBW and SGA are major clinical factors associated with these diseases in later life.3–5 Additionally, rapid weight gain in early infancy has been reported to be an important determinant of type 2 diabetes and cardiovascular disease in adulthood.6,7 Changes in body weight during such a sensitive developmental period are considered to have programming effects on the later diseases. In addition to these clinical predictors, quantitative biomarkers that reflect the fetal environment and potentially predict future cardiometabolic risks are currently required. In previous studies, cord blood biomarkers including leptin and adiponectin5,9 were reported to be associated with LBW or SGA. However, definitive biomarkers reflecting a poor fetal environment in early infancy have still not been established.

Cord blood glycoforms may serve as potential predictors of a poor fetal environment because glycosylation is one of the most important posttranslational modifications of proteins. In particular, N-glycan plays a crucial role in major human biological processes, such as cell–matrix interactions, protein folding, receptor binding, and protein clearance.10–12 Whereas
changes in serum N-glycans were considered to be novel diagnostic biomarkers of various diseases, especially different types of cancer.\textsuperscript{13,14} The highly complicated structures of N-glycans and difficulty in processing for their separation from complex mixtures hindered the progress of glycomics. However, emerging technologies have currently made it more feasible to acquire high throughput of glycome profiling. We developed the method of glycoblotting, which is a comprehensive automated N-glycan analysis using matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).\textsuperscript{15} The protocol requires only 5 to 10 μL of serum to quantitatively profile approximately 50 types of major N-glycans within 5 hours. The glycoblotting method has been used to detect specific N-glycans associated with different types of neoplastic diseases\textsuperscript{16–18} or non-neoplastic diseases.\textsuperscript{19,20} If specific N-glycans related to a poor fetal environment or rapid weight gain are detected, they may serve as biomarkers for predicting the incidence of future diseases, including type 2 diabetes and cardiovascular disease. In addition, the findings might provide novel insight into pathophysiological mechanisms of developmental origins of diseases.

In this study, we measured the glycosylation status in cord blood by using the MS-based glycoblotting method. The aim of the study is to evaluate associations between cord blood N-glycans and perinatal outcomes reflecting the fetal environment, such as LBW and SGA, or cord blood leptin and adiponectin. Furthermore, we conducted a prospective analysis and explored correlations of cord blood N-glycans and 6 or 18-month rapid weight gain after birth.

METHODS

Subjects from the Hamamatsu Birth Cohort

The study population was derived from the Hamamatsu Birth Cohort for Mothers and Children (HBC study). Details of the cohort setting were previously reported elsewhere.\textsuperscript{21} Briefly, we consecutively recruited pregnant women (N = 723) who were expected to give birth at 2 research sites located in Hamamatsu, Japan. The participants gave birth between December 2007 and May 2010, and were all Japanese. In a previous study, we established that the enrolled pregnant women were representative of the general population in Japan regarding age, socioeconomic status, and parity.\textsuperscript{21} The newborns in the cohort were also representative in birth weight and gestational age. From this cohort, we constructed a subcohort of 156 neonates. The subcohort consisted of 36 consecutive newborns with LBW (<2500 g) and 120 newborns with normal birth weight (NBW; 2750–3250 g). Both these groups of newborns, who were born at Hamamatsu University Hospital between February 2008 and December 2010, were included in the present study. The NBW subjects were randomly selected from the HBC after matching for maternal age. Subjects with multiple pregnancies were excluded. Baseline clinical data of each participant were extracted from the database of the HBC. In Japan, Maternal and Child Health Law encourages infants to undergo medical checkups at the ages of 1, 4, 6, 10, and 18 months. In the prospective exploratory analysis, we used data on weight from institutional medical checkups at these time points. The study was approved by the ethical committee of Hamamatsu University School of Medicine. The parents provided written informed consent to participate in the study.

Laboratory Methods

Cord blood samples were collected immediately after delivery. Serum samples were frozen below −80°C and stored until analysis. All procedures of N-glycan analysis were conducted using the SweetBlot (System Instruments, Hachioji, Japan), which is an automated machine for pretreatment and glycoblotting with a 96-well plate platform, according to previously reported procedures.\textsuperscript{15,17} Proteins in each 10-μL serum sample containing 40 pmol of an internal standard disialo-galactosylated biantennary N-glycan with amidated sialic acids (A2 amide glycan) were reduced and alkylated by 1,4-dithiothreitol (DTT) and iodoacetamide (Wako Pure Chemical Industries, Osaka, Japan). The mixture was then treated with trypsin and heat-inactivated. After being cooled to room temperature, the N-glycans were released from the tryptic glycopeptides by incubation with peptide N-glycanase F (New England BioLabs, Ipswich, MA) for 360 minutes at 37°C. After incubation, 20 μL of the solution was equivalent to 2.5 μL of serum, which was used for glycoblotting. The pretreated samples were mixed with 250 μL of BlotGlyco H beads (Sumitomo Bakelite, Co., Tokyo, Japan) to capture the N-glycans using stable hydrazide bonds. Acetyl capped of unreacted hydrazide groups on the beads was then carried out. After capping, on-bead methyl esterification of sialic acid carboxyl groups in the terminal of the captured N-glycans was then performed. The captured N-glycans were labeled by O-benzylhydroxylamine hydrochloride (BOA; Sigma-Aldrich, St Louis, MO) and eluted with 100 μL of water. BOA-labeled N-glycans were then released from the BlotGlyco H beads and detected using MALDI-TOF MS (Ultra-flex 3, TOF/TOF mass spectrometer, Bruker Daltonics, Bremen, Germany). All spectral conditions were obtained using the reflector mode, ions generated by Smartbeam (pulsed UV solid laser, λex = 355 nm, 50 Hz) with 25 kV as the acceleration voltage, 26.3 kV as the reflector voltage, 160 ns as pulsed ion extraction in the positive mode, and typically totaling 1000 shots of each spot. The intensity of the isotopic peak of each glycan was normalized using 40 μM of the internal standard (disialyl-octa-saccharide) for each status, and their concentrations were calculated from a calibration curve using human serum standards. Structures of N-glycans were determined according to the GlycoMod Tool (http://br.expasy.org/tools/glycomod).

Measurements of cord blood plasma levels of total, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) cholesterol, and triglycerides were performed by a high-sensitivity lipoprotein profiling system with high-performance liquid chromatography (Skylight Biotech Inc., Akita, Japan). Leptin and adiponectin levels were measured using commercial enzyme-linked immunoassay kits (R&D Systems, Minneapolis, MN; Otsuka Pharmaceutical, Tokyo, Japan, respectively).

Outcomes and Statistical Analysis

We determined outcomes indicating a poor fetal environment in cross-sectional analyses as follows. The primary outcomes were LBW and SGA. The secondary outcomes were levels of leptin and adiponectin in cord blood. SGA status was determined as a birth weight <10th percentile for gestational age according to standards developed by a study group of the Ministry of Health, Labour, and Welfare in Japan.\textsuperscript{22} Non-SGA status was defined as a birth weight ≥10th percentile for gestational age. In the prospective exploratory analysis, rapid weight gain after birth at the ages of 6 and 18 months were set as outcomes. Rapid weight gain was defined as the difference in SD score for weight at birth and that at each time point >0.67.
RESULTS

Clinical Characteristics of Mothers and Neonates

The clinical characteristics of the mothers and neonates are shown in Table 1. The proportions of cesarean sections, emergency cesarean sections, preterm, and SGA were significantly higher in the LBW group than in the NBW group. SGA was not observed in the NBW group. Mean gestational age and birth weight were significantly lower in the LBW group than in the NBW group. There were no significant differences in mean maternal or paternal age, and the proportions of ART, maternal smoking, first childbirth, and sex of the neonates.

Cord blood leptin and adiponectin levels in the LBW group were significantly lower than those in the NBW group. Mean total, LDL, and HDL cholesterol, and triglyceride levels in cord blood were not significantly different between the LBW and NBW groups. However, total, LDL, and HDL cholesterol levels tended to be lower in the SGA subjects than in the non-SGA subjects (61.1 ± 11.4 vs 66.3 ± 16.4 mg/dL, P = 0.070; 20.3 ± 6.63 vs 25.0 ± 9.30 mg/dL, P = 0.071; 30.1 ± 9.02 vs 34.4 ± 8.82 mg/dL, P = 0.071, respectively). By contrast, triglycerides tended to be higher in the SGA subjects compared with the non-SGA subjects (33.4 ± 11.7 vs 28.5 ± 14.1 mg/dL, P = 0.058).

Cross-sectional Analysis: Cord Blood N-Glycans and Perinatal Outcomes

Using MALDI-TOF MS analysis of glycoblotting, we identified 35 BOA-labeled N-glycans with a molecular weight (m/z) ranging from 1362.481 to 3865.407 (Supplemental Table, http://links.lww.com/MD/A862). Typical mass spectra of cord blood N-glycans in a newborn with LBW or NBW are shown in Figure 1. Among these 35 N-glycans, mean concentrations of cord blood N-glycans were not significantly different between the LBW and NBW groups. However, total, LDL, and HDL cholesterol levels tended to be lower in the SGA subjects than in the non-SGA subjects (61.1 ± 11.4 vs 66.3 ± 16.4 mg/dL, P = 0.070; 20.3 ± 6.63 vs 25.0 ± 9.30 mg/dL, P = 0.071; 30.1 ± 9.02 vs 34.4 ± 8.82 mg/dL, P = 0.071, respectively).

TABLE 1. Baseline Characteristics in Mothers and Neonates

|                          | LBW (n = 36) | NBW (n = 120) | P    |
|--------------------------|-------------|--------------|------|
| Maternal variables       |             |              |      |
| Age, years               | 31.5 (5.03) | 31.9 (4.93)  | 0.682|
| Advanced maternal age    | 8 (22.2 %)  | 38 (31.7 %)  | 0.276|
| Paternal age, years      | 33.4 (5.22) | 33.8 (6.01)  | 0.685|
| Assisted reproductive    | 4 (11.1 %)  | 13 (10.8 %)  | 0.963|
| Smoking during pregnancy | 2 (5.56 %)  | 10 (8.33 %)  | 0.583|
| Cesarean section, n (%)  | 19 (52.8 %) | 56 (46.7 %)  | 0.520|
| Emergency cesarean section, n (%) | 13 (36.1 %) | 22 (18.3 %) | 0.025|
| Neonatal variables       |             |              |      |
| Sex (female), n (%)      | 23 (63.9 %) | 79 (65.8 %)  | 0.830|
| Gestational age, weeks   | 37.6 (1.45) | 39.2 (1.06)  | <0.001|
| Preterm, n (%)           | 10 (27.8 %) | 2 (16.7 %)   | <0.001|
| Birth weight, g          | 2275.2 (194) | 2992 (131)  | <0.001|
| Birth weight SD score    | -1.81 (0.49) | -0.01 (0.32) | <0.001|
| Small for gestational age, n (%) | 17 (47.2 %) | 0 (0.0 %)   | <0.001|
| Cord blood variables     |             |              |      |
| Cholesterol              |             |              |      |
| Total, mg/dL             | 69.1 (19.2) | 64.8 (14.9)  | 0.266|
| LDL, mg/dL               | 26.2 (11.1) | 23.6 (8.09)  | 0.330|
| HDL, mg/dL               | 33.6 (9.67) | 33.9 (8.67)  | 0.847|
| Triglycerides, mg/dL     | 29.5 (11.7) | 29.0 (14.8)  | 0.873|
| Leptin, ng/mL            | 1.62 (1.38) | 4.18 (3.12)  | <0.001|
| Adiponectin, µg/mL       | 26.6 (10.6) | 31.2 (12.3)  | 0.038|

Data are expressed as the mean (SD) or n (%).

HDL = high-density lipoprotein, LBW = low birth weight, LDL = low-density lipoprotein, NBW = normal birth weight, SD = standard deviation.
G1956 and G2220 values also tended to be lower in the LBW group than in the NBW group, but this did not reach statistical significance. Unlike other N-glycans, the mean G2525 value was significantly higher in the LBW group than in the NBW group. There was no significant difference in the total amount of N-glycans between the groups.

We then conducted logistic regression to evaluate associations between these candidate N-glycans and LBW. Odds ratios of LBW for these candidate N-glycans in logistic regression models are shown in Table 3. In the model adjusted for maternal smoking, first childbirth, sex of the neonate, and ART, G1915, G2744, G3049, G3414, and G3719 showed significant inverse associations with LBW. The significance of the association between G1956 or G2525 and LBW was borderline in the multivariate model. In contrast to other N-glycans, G2525 only tended to have a positive association with LBW. We found that SGA was inversely associated with G3414 in logistic regression adjusted for maternal smoking and ART (Table 3). Of the N-glycans associated with LBW, only G3414 was significantly correlated with SGA.

Associations of cord blood N-glycans and leptin or adiponectin were assessed in multiple regression analyses adjusted
**TABLE 3. Logistic Regression Analysis of Associations Between N-Glycans and LBW, SGA or Rapid Weight Gain**

| N-Glycans       | m/z    | OR (95% CI)   | P       |
|-----------------|--------|---------------|---------|
| Cross-sectional analyses |        |               |         |
| Low birth weight |        |               |         |
| G1915           | 1914.698 | 0.62 (0.39–0.97) | 0.035   |
| G1956           | 1955.724 | 0.39 (0.14–1.03) | 0.058   |
| G2220           | 2219.809 | 11.5 (0.78–181.0) | 0.076   |
| G2525           | 2524.920 | 1.23 × 10⁻⁵ (1.8 × 10⁻¹⁰–0.09) | 0.010   |
| G2744           | 2743.994 | 5.1 × 10⁻³ (6.4 × 10⁻⁸–0.01) | <0.001  |
| G3049           | 3049.105 | 0.04 (2.5 × 10⁻³–0.39) | 0.004   |
| G3414           | 3414.238 | 0.01 (7.6 × 10⁻⁴–0.15) | <0.001  |
| Small for gestational age |        |               |         |
| G3414           | 3414.238 | 0.04 (4.1 × 10⁻⁴–0.92) | 0.043   |
| Prospective exploratory analyses |        |               |         |
| Rapid weight gain at 6 months after birth |        |               |         |
| G1915           | 1914.698 | 0.34 (0.16–0.64) | 0.002   |
| G2220           | 2219.809 | 0.26 (0.08–0.69) | 0.006   |
| G2744           | 2743.994 | 1.23 × 10⁻⁵ (1.10 × 10⁻¹²–0.10) | 0.016   |
| G3049           | 3049.105 | 6.17 × 10⁻⁷ (1.86 × 10⁻⁸–0.04) | 0.002   |
| G3414           | 3414.238 | 9.98 × 10⁻⁵ (2.99 × 10⁻⁶–0.18) | 0.001   |
| G3719           | 3719.349 | 0.08 (0.01–0.49) | 0.006   |
| Rapid weight gain at 18 months after birth |        |               |         |
| G1915           | 1914.698 | 0.51 (0.27–0.92) | 0.025   |
| G1956           | 1955.724 | 0.34 (0.09–1.17) | 0.089   |
| G2074           | 2073.751 | 0.02 (8.11 × 10⁻⁵–1.70) | 0.084   |
| G2744           | 2743.994 | 114.3 (0.66–3.67 × 10⁴) | 0.074   |
| G3049           | 3049.105 | 2.43 × 10⁻⁶ (4.37 × 10⁻¹⁵–6.22 × 10⁻³) | 0.004   |
| G3414           | 3414.238 | 3.48 × 10⁻⁶ (3.61 × 10⁻¹⁰–4.94 × 10⁻⁷) | <0.001  |
| G3719           | 3719.349 | 0.02 (4.42 × 10⁻⁴–0.29) | 0.004   |

**CI** = confidence interval, **LBW** = low birth weight, **OR** = odds ratio, **SGA** = small for gestational age.

1 Associations of N-glycans and LBW were adjusted for maternal smoking, ART, first childbirth, and the neonate’s sex.

2 Associations of N-glycans and SGA were adjusted for maternal smoking and ART.

3 Associations of N-glycans and rapid weight gain were adjusted for maternal smoking and ART.
 Prospective Exploratory Analysis: Cord Blood N-Glycans and Rapid Weight Gain After Birth in Early Infancy

The 18-month prospective follow-up data of body weight are shown as actual data and SD scores in Table 4. The proportion of rapid weight gain until the age of 6 or 18 months was significantly higher in the LBW group than in the NBW group. Associations between cord blood N-glycans and rapid weight gain at 6 or 18 months after birth are shown in Table 3. G1915, G2744, G3049, G3414, and G3719, which were N-glycans that were associated with LBW, were significantly inversely correlated with rapid weight gain at 6 or 18 months. G2220 was inversely associated with rapid weight gain at 6 months after birth. G1956 and G2074 tended to have inverse associations with rapid weight gain at 18 months after birth. In contrast to other N-glycans, G2439 trended to positively associated with rapid weight gain at 18 months after birth.

DISCUSSION

The present study is the first to evaluate glycosylation status in cord blood and showed that different types of N-glycans were correlated with LBW and SGA or rapid weight gain in early infancy. Of the 35 N-glycans that were identified by glycoblotting, G1915, G2744, G3049, G3414, and G3719, which were N-glycans that were associated with LBW, were significantly inversely associated with rapid weight gain at 6 or 18 months. G2220 was inversely associated with rapid weight gain at 6 months after birth. G1956 and G2074 tended to have inverse associations with rapid weight gain at 18 months after birth. In contrast to other N-glycans, G2439 trended to be positively associated with rapid weight gain at 18 months after birth.

TABLE 4. Follow-up Data of Body Weight After Birth

| Time after birth | Actual data, kg | n | LBW | n | NBW | P |
|------------------|----------------|---|-----|---|-----|---|
| 1 months         | 30             | 6.03 (0.60) | 42 | 4.19 (0.37) | <0.001 |
| 4 months         | 30             | 7.08 (0.91) | 21 | 7.51 (0.86) | 0.176 |
| 6 months         | 28             | 7.68 (0.75) | 39 | 8.49 (0.84) | 0.002 |
| 10 months        | 28             | 9.50 (0.85) | 37 | 10.0 (1.02) | 0.040 |
| 18 months        | 28             | 7.18 (0.79) | 39 | 8.49 (0.84) | 0.002 |
| SD score         | 1 months       | -1.28 (0.74) | 42 | 0.07 (0.66) | <0.001 |
| 4 months         | 30             | -0.90 (0.74) | 40 | -0.11 (0.73) | <0.001 |
| 6 months         | 13             | -0.77 (1.07) | 21 | -0.08 (0.99) | 0.067 |
| 10 months        | 28             | -0.98 (0.79) | 39 | -0.18 (0.96) | <0.001 |
| 18 months        | 28             | -0.59 (0.79) | 37 | -0.01 (1.00) | 0.015 |
| Rapid weight gain| Birth to 6 months, n (%) | 30 | 23 (76.7%) | 40 | 8 (20.0%) | <0.001 |
| Birth to 18 months, n (%) | 31 | 22 (71.0%) | 38 | 9 (23.7%) | <0.001 |

Data are expressed as the mean (SD) or n (%).
LBW = low birth weight, NBW = normal birth weight, SD = standard deviation.
(G2744, G3049, G3414, and G3719) are associated with LBW. These N-glycans may be derived from AFP without fucose, which is produced by the fetus. AFP without fucose is produced by the yolk sac or fetal liver, and production gradually increases during pregnancy. Therefore, N-glycans of AFP without fucose might be sensitive markers for gestational age. In another recent study, G3049, G3414, and G3719 were also significant predictive factors of castration-resistant prostate cancer.17 This finding suggests that similar pathways of glycosylation exist in a poor fetal environment and castration-resistant prostate cancer.

Among the outcomes in our study, leptin and adiponectin, which are members of adipokines secreted from adipose tissue, were positively related to the fetal concentrations of N-glycans. Additionally, both adipokines were associated with a large number of N-glycans (leptin: 9 out of 35 N-glycans, adiponectin: 14 out of 35 N-glycans). These findings indicate that serum glycosylation status has a strong correlation with these adipokines. Future studies need to be performed for assessing correlations of N-glycans and metabolic status because leptin and adiponectin regulate energy balance and insulin sensitivity.24

Our study has some limitations. The proportion of the subjects who dropped out in the prospective exploratory analysis was relatively high. There may be selection bias in this analysis, although associations of the 5 candidate N-glycans and outcomes were almost consistent between the cross-sectional analysis and the prospective exploratory analysis. In addition, given the observational nature of this study, the findings alone could not prove causal associations of N-glycans with a poor fetal environment and rapid weight gain in early infancy. Further investigation of the molecular mechanism would elucidate the causal associations of these conditions. In this study, we did not examine the thresholds of N-glycans (cut-off values) because the outcomes in the study including LBW, SGA and rapid weight gain, were not diseases themselves. Future studies need to evaluate associations of N-glycans and cardiometabolic diseases, which should be true endpoints, and also investigate the thresholds of cord blood N-glycans. There are also unavoidable limitations of laboratory methods in this study. Cord blood samples were collected immediately after delivery, and serum samples were frozen below −80°C and stored until analysis. However, in the clinical study protocol, the time intervals of blood sampling, serum generation, and freezing were not planned to be measured. In some clinical settings (eg, emergency settings), precisely measuring these time intervals is difficult. Therefore, the time intervals could not be determined in this study, unlike laboratory settings. Additionally, we do not have the information on glycosylation/sialylation status (half-life) in cord blood. However, stability of N-glycan profiles in human plasma has been established and serum N-glycan profiles have been widely used as potential biomarkers of various diseases.13,14

In conclusion, our comprehensive analyses on the N-glycan status in cord blood shows that G1915, G2744, G3049, G3414, and G3719 are potentially useful N-glycans for predicting a poor fetal environment and rapid weight gain in early infancy, both of which are clinical determinants of future type 2 diabetes and cardiovascular disease. These N-glycans are structurally categorized into 2 different categories. The findings of the present study suggest that mass spectrometry-based quantitative cord blood glycoform profiling is an informative method for predicting a poor fetal environment and rapid weight gain. Future studies assessing the potential mechanism of developmental origins of diseases including type 2 diabetes and cardiovascular disease should also benefit from the findings in the present study.

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