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Relationship of folate, vitamin B_{12} and methylation of insulin-like growth factor-II in maternal and cord blood

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Abstract

Background/Objective—One of the speculated mechanisms underlying fetal origin hypothesis of breast cancer is the possible influence of maternal environment on epigenetic regulation, such as changes in DNA methylation of the insulin-like growth factor-2 (*IGF2*) gene. The aim of the study is to investigate the relationship between folate, vitamin B_{12} and methylation of the *IGF2* gene in maternal and cord blood.

Subjects/Methods—We conducted a cross-sectional study to measure methylation patterns of *IGF2* in promoters 2 (P2) and 3 (P3).

Results—The percentage of methylation in *IGF2* P3 was higher in maternal blood than in cord blood ($p<0.0001$), while the methylation in P2 was higher in cord blood than in maternal blood ($p=0.016$). P3 methylation was correlated between maternal and cord blood ($p<0.0001$) but not P2 ($p=0.06$). The multivariate linear regression model showed that methylation patterns of both promoters in cord blood were not associated with serum folate levels in either cord or maternal blood, while the P3 methylation patterns were associated with serum levels of vitamin B_{12} in mother's blood (MC=−0.22, $p=0.0014$). Methylation patterns in P2 of maternal blood were associated with serum levels of vitamin B_{12} in mother's blood (MC=−0.23, $p=0.012$), exposure to passive smoking (MC=0.46, $p=0.034$) and mother's weight gain during pregnancy (MC=0.23, $p=0.019$).

Conclusions—The study suggests that environment influences methylation patterns in maternal blood, and then the maternal patterns influence the methylation status and levels of folate and vitamin B_{12} in cord blood.

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Keywords

Folate; vitamin B_{12}; methylation; IGF2; cord blood

INTRODUCTION

Trichopoulos proposed in 1990 that breast cancer might originate *in utero* (Trichopoulos, 1990). This hypothesis is supported by epidemiological studies showing a strong association between breast cancer risk and various birth phenotypes such as birth weight, birth length, and gestational age as reviewed by Michels and Xue (Michels & Xue, 2006). One of the speculated mechanisms underlying the relationship between birth outcomes and breast cancer risk is the possible influence of maternal environment on epigenetic regulation, such as changes in DNA methylation of the insulin-like growth factor-2 (*IGF2)* gene (Michels & Xue, 2006).

IGF2 is an imprinted gene in which methylation silences the maternal allele resulting in only one parental allele (the paternal allele) to be expressed. Loss of imprinting (LOI) of *IGF2* leads to biallelic expression and increases production of IGF2. In the transgenic mouse model, overexpression of *IGF2* can induce mammary cancer (Bates et al., 1995). Human studies have found increased *IGF2* expression in both breast tumors(van Roozendaal et al., 1998; Wu et al., 1997) and other breast lesions (McCann et al., 1996). *IGF2* expression is known to be an essential determinant of fetal growth (Ong et al., 2000; Sun et al., 1997). It is plausible that LOI of *IGF2* may result in high intrauterine expression of IGF2, which increases the number of susceptible stem cells in the mammary gland or enhances breast cell transformation *in utero*, prior to puberty when the tissues are very sensitive to carcinogenic insults (Michels & Xue, 2006). LOI of *IGF2* (DNA hypomethylation) has only been investigated in adults, but no studies have evaluated IGF2 methylation in newborn infants.

Recent evidence shows that DNA methylation patterns are reprogrammed genome-wide *in utero*, which result in stable changes in gene expression that may be maintained throughout a person's lifespan (Wu et al., 2004). The genomic imprints are established during the development of germ cells into sperms and eggs. After fertilization, the genomic imprints go through a resetting process, genome-wide demethylation after fertilization and *de novo* methylation after implantation(Reik & Walter, 2001). While the underlying mechanisms for the *de novo* methylation *in utero* have yet to be fully established, maternal nutrition intake has been demonstrated to play a critical role in DNA methylation *in utero*. For example, studies of *in vitro* manipulation of human embryos have shown that environmental exposures during *in vitro* manipulation of early embryo induce DNA methylation alternations at imprinted loci, resulting in subsequent human diseases(Cox et al., 2002; DeBaun et al., 2003; Orstavik et al., 2003). Using the Avy/a mice model, Wolff et al.(Wolff et al., 1998) found that the coat-color distribution of offspring born to dams supplemented with folic acid, vitamin B_{12}, choline, and betaine was shifted toward the brown phenotype compared to those born to non-supplemented dams through increasing Avy methylation in the offspring(Waterland & Jirtle, 2003). In addition, maternal dietary exposure to folate

alters the effects of adverse maternal environmental exposures on the DNA methylation changes of specific genes(Lillycrop et al., 2005).

One carbon metabolism, which ultimately provides the methyl groups for all DNA methylation reactions, is highly dependent on dietary methyl donors such as folate/folic acid and betaine and cofactors such as vitamins B_{12}, B_6, and B_2, and zinc (Van den Veyver, 2002). Very few studies have been conducted to investigate whether maternal nutrition influences an offspring's DNA methylation patterns (Steegers-Theunissen et al., 2009). As such, we conducted a pilot study to examine the DNA methylation pattern of *IGF2* in cord blood and to examine whether maternal serum levels of folate and vitamin B_{12} influence IGF2 methylation pattern in cord blood.

SUBJECTS AND METHODS

Study Population

A pilot study was conducted in Houzhai Center Hospital in Zhengzhou, China. Pregnant women, who were 19 years of age or older without pre-existing medical conditions such as diabetes, hepatitis B infection, or other coeliac diseases and who came to the hospital for delivery between April and September in 2008, were considered eligible for the study. All of the participants were from suburb close to Zhengzhou city. A total of 257 potentially eligible subjects were identified during the study time period. We reached potential eligible subjects who delivered babies from Mondays to Thursdays, which yield a total of 120 mothers. Among those, 99 (82.5%) were participated in the study with both maternal and cord blood available. Upon receiving their written consent, an in-person interview was conducted at the hospital using a standardized and structured questionnaire to collect information on demographic factors, maternal stature, medical conditions and medication use including supplemental vitamins, reproductive history, smoking and alcohol consumption, and dietary intakes. Two 5 ml fasting blood samples were provided by the subjects at the hospital before delivery. Blood was collected in red top vacuum tubes, and placed immediately on ice. After centrifugation, serum and white blood cells were separated and frozen at −70°C for subsequent analyses. Cord blood samples were also collected and processed using the same protocol. The birth weight, birth length and head circumference, and gestational age of newborns were abstracted from delivery room records. The study was approved by the Institutional Review Board at Zhengzhou University, China.

IGF2 Methylation Analysis

Genomic DNA was extracted from blood samples (mother's blood and cord blood) using AxyPrep Blood Genomic DNA Miniprep kit (Axygen Biosciences, USA). The DNA samples were treated with sodium bisulfite using the EZ DNA Methylation-Gold™ kit (Zymo Research). CpG site methylation within two promoter regions of the IGF-II gene, P2 and P3, was analyzed using a Power SYBR Green PCR Master Mix (Applied Biosystems, UK) and real-time methylation specific PCR (qMSP) (MX3000, USA). Two qMSP assays, P2A and P3A, were developed to evaluate methylation in P2 and P3 regions (Beeghly et al., 2007). Each qMSP assay included two pairs of PCR primers; one for methylated and one for unmethylated bisulfite converted sequences. Primer sequences were referenced to the report

by Beeghly et al. (Beeghly et al., 2007) and were listed in Table 1, along with annealing temperatures and product sizes. In table 1, the bold letters indicate bisulfite conversion of C to T.

In the PCR reaction (20 μL), 1 μL of bisulfite-treated DNA template with a proximate concentration of 100ug/ml was mixed with 10 μL of 2×Power SYBR Green PCR Master Mix (Applied Biosystems) and a pair of primers in a final concentration of 200 nmol/L. The PCR conditions included denaturing at 95 C for 10 min, and 40 cycles of denaturing at 94 C for 15s, annealing at 58 C or 59 C for 30s and extension at 72 C for 30s. After PCR amplification, a dissociation curve was generated to confirm the size of PCR product. Four quality control (QC) samples was included in each batch of methylation analysis, including two negative QC samples from untreated DNA and two water without DNA template.

Serum Folate Analysis

Serum folate was determined using a Folate Reagent kit (A14028, Beckman Coulter) and the paramagnetic particle chemiluminescent immunoassay system Access 2 analyzer (BECKMAN COULTER, USA). A standard curve was generated using the six calibrators included in the kit. All of the materials and reagents used for the test were provided by the Beckman Coulter Ins. We included one negative QC sample (contain human serum albumin buffer only) in each batch and one repeat in every 10 samples. The coefficients of variation (CV) for within- and between-run were 0.51 and 2.33% respectively.

Serum Vitamin B_{12} Analysis

Serum vitamin B_{12} was determined using a B_{12} Reagent kit (A911973) and the paramagnetic particle chemiluminescent immunoassay system Access 2 analyzer (BECKMAN COULTER, USA). A standard curve was made by six calibrators which had vitamin B_{12} concentrations of 0, 106, 244, 493, 885, and 1,500pg/ml. All of the assay materials and reagents were provided by the Beckman Coulter Ins. We included one negative QC sample (contain human serum albumin buffer only) in each batch and one repeat in every 10 samples. The coefficients of variation (CV) for within- and between-run were 0 and 2.0% respectively.

Statistical Analysis

The methylation level was presented as a percentage of methylated sequences in all analyzed DNA sequences which was calculated based on the formula: $M/(M+U) \times 100\%$, where M is the copy number of methylated sequences and U is the copy number of unmethylated sequences. The above formula can be further expressed as $1/(1+U/M) \times 100\% = 1/[1+2^{-(\Delta Ct)}] \times 100\%$, where ΔCt = Ct(U)-Ct(M). Ct is the threshold of PCR cycle number at which the increase in fluorescent signal reaches a critical point.

Levels of methylation at each promoter region were logarithmically-transformed to achieve approximately normal distribution and the transformed values were used in data analyses. The paired t-test was employed to compare serum levels of folate, vitamin B_{12}, and methylation percentages between matched cord blood and maternal blood. Separate

multivariate linear regression models were used to estimate the mean change (MC) in methylation percentages of IGF2 in cord blood in relation to an increase of one standard deviation in serum levels of folate and vitamin B_{12} in cord blood and mother's blood, and various maternal and neonatal characteristics including maternal age, maternal pre-pregnancy body mass index (BMI), maternal exposure to passive smoking (no active smoking in the study population), maternal education, maternal weight gain during pregnancy of index, parity, supplement intake during pregnancy of index, birth weight, birth length, gestational age, and baby's gender. The standard deviation for each characteristic was calculated from the entire study population. Multivariate linear regression model was also employed to estimate the MC in methylation percentages of IGF2 in mother's blood for an increase of one standard deviation in serum levels of folate and vitamin B_{12} in mother's blood, maternal age, maternal pre-pregnancy body mass index (BMI), maternal exposure to passive smoking, maternal education levels, maternal weight gain during pregnancy of the index, parity, and supplement intake during pregnancy of index. All statistical tests were two sided with 0.05 as a cutoff for significance. Statistical analyses were performed using the SAS software, version 9.1 (SAS Institute, Inc., Cary, NC).

RESULTS

As shown in Table 2, there were 99 newborns enrolled in the study (52 boys and 47 girls); of them, three had low birth weight (<2,500g) and one was a preterm birth (<37weeks). Forty percent of women in the study delivered by cesarean section and one third of them were not on their first parity. The mean maternal age was 27.8 years. A total of 13 women had BMI greater than 25 before pregnancy. About 30% women were exposed to passive smoking and 53% took supplementation (mainly includes calcium and traditional Chinese medicine) during pregnancy.

The mean levels of serum folate and vitamin B_{12} were 7.29ng/ml (range=0–21.0ng/ml) and 240pg/ml (range=78–1500ng/ml) in cord blood and 2.29ng/ml (range=0–11.2ng/ml) and 175pg/ml (range=57–1120ng/ml) in mother's blood respectively. The serum folate and vitamin B_{12} levels in cord blood were significantly higher than those in mother's blood ($p<0.0001$). The serum levels of folate and vitamin B_{12} in cord blood and maternal blood were significantly correlated to each other (Pearson Correlation Coefficient=0.37, P=0.0003 for folate; Pearson Correlation Coefficient=0.31, P=0.0021 for vitamin B_{12}).

The mean percentages of methylation in IGF2 promoter 3 (P3) were 5.83% (SD=4.92%, range=0.87–34.97%) for maternal blood and 4.11% (SD=3.04%, range=0.48–18.1%) for cord blood, and the percentage of methylation was significantly higher in maternal blood than in cord blood ($p<0.0001$). In contrast, the mean percentages of methylation in IGF2 promoter 2 (P2) were 2.85% (SD=1.78%, range=0.11–10.1%) in maternal blood and 3.54% (SD=2.80%, range=0.53–18.7%) in cord blood, and the methylation percentage was significantly higher in cord blood than in maternal blood ($p=0.016$).

The mathylation patterns of P3 was significantly correlated between maternal and cord blood (Pearson Correlation Coefficient=0.41, P<0.0001). Similar correlation, however, was not observed for P2 (Pearson Correlation Coefficient=0.19, P=0.06). The observed

correlations were not changed when the analyses were stratified by maternal serum levels of folate. After stratification by maternal serum levels of vitamin B_{12}, we found significant correlations of methylation patterns between maternal and cord blood for both P3 (Pearson Correlation Coefficient=0.43, P=0.0001) and P2 (Pearson Correlation Coefficient=0.27, P=0.02) when the levels were equal to or greater than 200pg/ml. No significant correlations were observed when maternal serum levels were equal to or greater than 200pg/ml.

Methylation of *IGF2* promoters in cord blood were not associated with serum levels of folate in either cord blood or mother's blood (Table 3). P3 methylation in cord blood appeared to be inversely associated with serum levels of vitamin B_{12} in maternal blood (MC=−0.22, p=0.0014) but not in cord blood (MC=−0.042, p=0.60). The association was diminished when the analyses were restricted to those who had maternal serum levels of folate ≥ 3ng/ml or vitamin B_{12} ≥ 200pg/ml (data not shown). No association with methylation in cord blood was found for maternal and neonatal characteristics.

P2 methylation in maternal blood were associated with mother's vitamin B_{12} levels (MC= −0.23, p=0.012), exposure to passive smoking (MC=0.46, p=0.034) and weight gain during pregnancy (MC=0.23, p=0.019 Table 3), while no significant association was observed for P3 methylation in mother's blood.

DISCUSSION

The *IGF2* gene contains four promoters (P1–P4) located upstream of exons 1, 4, 6, and 7 respectively (Engstrom et al., 1998). Each of the promoters can initiate transcription giving rise to a family of transcripts with distinct 5′-untranslated regions (van Dijk et al., 1991). Since these transcripts differ in their translatability and stability (Holthuizen et al., 1993; Nielsen & Christiansen, 1992; Nielsen & Christiansen, 1995), the resulting IGF2 protein may vary in terms of quantity. The usage of *IGF2* promoters has been shown to be both temporal and tissue specific with P2–P4 active in all fetal tissues and P1 only active in the healthy adult liver (Holthuizen et al., 1993). While the first promoter (P1) contains few CpG sites, the latter three promoters (P2–P4) exist in a large CpG rich region (Beeghly et al., 2007; Issa et al., 1996). Because the P4 has been found to have the lowest frequency of methylation among the three promoters in our previous study (Beeghly et al., 2007), we focused on P2 and P3 promoters in this pilot study.

We found a strong correlation of P3 methylation between maternal and cord blood, but the correlation was diminished when the maternal serum levels of vitamin B_{12} were within the normal range. A strong correlation of P2 methylation between maternal and cord blood was observed only among those who had maternal serum levels of vitamin B_{12} lower than the normal range. These results might suggest a stronger heritable nature of methylation when maternal vitamin B_{12} was low. In another word, higher maternal vitamin B_{12} could alter inherited methylation patterns. Because the maternal serum levels of folate and vitamin B_{12} in this study population was relatively low with 71% of the population having serum levels of folate less than 3ng/ml and 75% of the population having vitamin B_{12} less than 200pg/ml, the sample size were limited in the stratified analyses particularly for those who had higher levels.

In this pilot study, the methylation patterns of P3 in cord blood were negatively associated with serum levels of vitamin B_{12} in maternal blood. No association was observed between methylation patterns and serum folate levels in either mother's blood or cord blood. The higher percentages of population had normal concentration in cord blood for folate than for vitamin B_{12} (83% vs. 35%) might be a possible explanation. In addition, the levels of folate and vitamin B_{12} were much higher in cord blood than in mother's blood suggesting that the enrichment of necessary one carbon nutrients ensures the development of infants even in a very low concentration. On the other hand, the inconsistent findings for P2 and P3 in cord blood may suggest that influence on *de novo* methylation is likely to be promoter or CpG site dependent.

The methylation patterns in mother's blood were negatively associated with serum levels of vitamin B_{12} but not folate, and the association was only observed for P2. We also observed that mother's *IGF2* P2 methylation status was associated with exposure to passive smoking and weight gain during pregnancy. Weight gain during pregnancy reflects complex endogenous and exogenous factors such as nutrition, energy intake, physical activity, individual's metabolic rate, and certain underlying diseases, etc. It is difficult to delineate specific endogenous or environmental factors underlying the observed association. However, the results suggest that mother's methylation status can be affected by environmental factors.

One of the limitations of the study is that none of the study subjects took folic acid supplements before or during pregnancy, which limited our ability to examine the effect of folic acid supplementation. Since serum folate and vitamin B_{12} concentrations reflect the balance of folate and vitamin B_{12} intake from diet and other resources, their absorption and excretion, using serum concentrations is more reliable compared to using the data of dietary intake from questionnaire. Since the majority of study participants had serum levels of folate or vitamin B_{12} in the lower end of the normal range (defined as <3ng/ml for folate and 200pg/ml for vitamin B_{12}), which is comparable with the levels in populations who lived in the areas without food fortification program (Lehti, 1989; Thoradeniya et al., 2006; Vobecky et al., 1985). As such, the results of our study may not be generalizable to populations with high levels of folate or vitamin B_{12}.

DNA methylation patterns are tissue specific. DNA derived from peripheral blood cells are mainly from neutrophils, eosinophils, lymphocytes, and monocytes, which are likely to have different methylation profiles from those of other tissues (Moverare-Skrtic et al., 2009). The inconsistent results between maternal blood and cord blood observed in the current study could be due to different composition of individual celltypes between maternal and cord blood. It is important that celltypes are controlled in future studies.

In conclusion, the study provides the first human evidence that heritable nature of and environmental influences on methylation patterns are not uniform across different promoters within the same gene. Given the lack of understanding of methylation status in newborns and factors that influence *de novo* methylation, studies using a genome-wide approach to identify DNA methylation patterns of CpG islands in newborns and factors that are responsible for *de novo* methylation are needed.

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Table 1

MSP primer sequences, annealing temperatures, and product size summary

prime* r	Primer sequence 5′→3′	T_a(°C)	Product(bp)
*P*2A, *M*, *F*	CGGA**TTTTTTT**ACGCGGGGA**T**CG	59	127
*P*2A, *M*, *R*	CCGA**A**CTTAA**A**CG**AA**CGTCCG		
*P*2A, *U*, *F*	G**TT**TG**T**GGA**TTTTTTT**A**T**G**T**GGGGA**TT**G		137
*P*2A, *U*, *R*	**A**CA**M**CCC**A**AA**A**CTTA**ACAAACA**TCC**A**		
*P*3A, *M*, *F*	CG**T**AG**T**CGG**TT**TTT**T**CGCGCG	58	149
*P*3A, *M*, *R*	CG**AA**CG**AAA**CGCGCA**A**CCG		
*P*3A, *U*, *F*	**T**GG**T**GT**T**AG**TT**GG**TT**TTT**TT**G**T**G**T**GTT		159
*P*3A, *U*, *R*	CCT**AAA**CAC**AAACAAAA**CA**C**A**C**AA**CC**A**		

*Primer name: *P* means promoter region, *M* methylated, *U* unmethylated, *F* forward, *R* reverse

Table 2

Selected characteristics of mothers and newborns (n=99)

Newborn's characteristics		Number	Mean (Standard Deviation)	Range
Birth weight	<2,500g	3		
	2,500g–4,000g	93	3,200 (400)	2,100–4,300
	>4,000g	3		
Birth length	<50cm	42		
	50cm	28	49.5 (1.9)	42–53
	>50cm	29		
Head circumference	<34cm	30		
	34cm	47	33.8(1.2)	29–38
	>34cm	22		
Gestational age	<37weeks	1		
	37–41weeks	96	39.1 (1.1)	36–43
	≥42weeks	2		
Gender	Boy	52		
	Girl	47		
Caesarean birth	Yes	41		
	No	58		

Mother's characteristics		Number	Mean (Standard Deviation)	Range
Age	<25 years	35		
	25–29 years	25		
			27.8 (5.3)	19–37
	30–34 years	29		
	35+ years	10		
Height	<160cm	44		
	160–164cm	34	159.5(5.1)	140–170
	165cm+	21		
Pre-pregnancy BMI	<20	37		
	20–25	49	21.4(2.6)	14.7–28.3
	>25	13		
Weight gain during pregnancy	<10kg	9		
	10–20kg	85	14.6 (3.6)	5–22.5
	>20kg	5		
Highest education level	Elementary school	18		
	Middle school	57		
	High school	19		
	College or graduate school	5		
Passive smoking	Yes	30		
	No	69		
Number of live births[1]	1	68		
	2	26		

Mother's characteristics		Number	Mean (Standard Deviation)	Range
	>2	5		
Supplementation intake[2]	Yes	53		
	No	46		

[1]The number of live births included the current birth.

[2]The supplements included calcium and the traditional Chinese medicine.

Table 3

Associations between methylation status and serum folate and vitamin B_{12} levels

	MC[1]	P-value
Cord blood[2,3]		
Promoter P2		
Cord blood serum folate	0.18	0.065
Cord blood serum vitamin B_{12}	−0.027	0.75
Maternal blood serum folate	0.052	0.47
Maternal blood serum vitamin B_{12}	0.094	0.19
Promoter P3		
Cord blood serum folate	−0.027	0.77
Cord blood serum vitamin B_{12}	−0.042	0.60
Maternal blood serum folate	0.049	0.47
Maternal blood serum vitamin B_{12}	−0.22	0.0014
Mother's blood[4]		
Promoter P2		
Maternal serum folate	0.13	0.17
Maternal serum vitamin B_{12}	−0.23	0.012
Maternal exposure to passive smoking	0.46	0.034
Weight gain during pregnancy	0.23	0.019
Promoter P3		
Maternal serum folate	0.13	0.15
Maternal serum vitamin B_{12}	−0.096	0.25

[1] Mean change per standard deviation of each characteristic.

[2] The multivariate linear regression model also included mother's age, maternal pre-pregnancy BMI, weight gain during pregnancy, mother's highest education level, parity, supplementation intake during pregnancy, baby's birth weight and birth length, baby's gender, and gestational age.

[3] Separated models were run for the folate and vitamin B_{12} levels in maternal and cord blood.

[4] The multivariate linear regression model also included mother's age, mother's pre-pregnancy BMI, mother's highest educational level, parity, and supplementation intake during pregnancy.