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Low Maternal Dietary Folate Alters Retrotranspose by Methylation Regulation in Intrauterine Growth Retardation (IUGR) Fetuses in a Mouse Model

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Background:

Maternal folate deficiency-mediated metabolic disruption is considered to be associated with the risk of intrauterine growth retardation (IUGR), but the exact mechanism remains unclear. The retrotransposon long interspersed nucleotide element-1 (LINE-1), which can induce birth defects via RNA intermediates, plays crucial roles during embryonic development. We investigated potential relationships between maternal folate and DNA methylation, and possible roles of LINE-1 in IUGR.

Material/Methods:

The IUGR model was established by feeding female mice 1 of 3 diets – control diet (CD), folate-deficient diet for 2 weeks (FD2w), and folate-deficient diet for 4 weeks (FD4w) – prior to mating. Maternal serum folate, 5-methyltetrahydrofolate (5-MeTHF), S-adenosylmethionine (SAM), and S-adenosylhomocysteine (SAH) concentrations and global DNA methylation were assessed by LC/MS/MS method. LINE-1 methylation levels in fetuses were examined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. LINE-1 expression levels were validated by real-time PCR.

Results:

Maternal folate deficiency caused plasma folate and 5-MeTHF levels to decrease and SAH level to increase in the FD4w group. Compared with the CD group, methylation levels of genomic DNA and LINE-1 decreased significantly in placenta and fetal tissues from the FD4w group. Expression of LINE-1 open reading frame 1 (ORF1) protein was elevated in fetal liver tissues. Furthermore, a strong correlation was found between methylation and disrupted one-carbon metabolism, implying that dietary folate plays important roles during embryogenesis.

Conclusions:


Maternal dietary folate deficiency impaired one-carbon metabolism, leading to global DNA and LINE-1 hypomethylation, and then increased retrotransposition in fetuses, which can lead to IUGR.

MeSH Keywords:

DNA Methylation • Fetal Growth Retardation • Folic Acid • Long Interspersed Nucleotide Elements

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Background

Intrauterine growth retardation (IUGR) is defined as a fetal birth in the 10th percentile, or at least 2 standard deviations (SDs) below the mean of other fetuses of the same gestational age [1]. IUGR carries both short- and long-term disadvantages for the offspring, including an increased risk of fetal, neonatal, and infant death, impaired postnatal growth, defective immune function, and impaired intellectual development [2,3]. The pathogeny of IUGR is complicated.

Proper maternal nutrition during gestation is crucial to embryonic development and fetal growth. Some studies [4–6] have shown that inadequate maternal intake of nutrients such as folate, vitamins, and minerals at this stage can result in deleterious consequences, and these types of deficiencies are thought to be a major factor in the etiology of IUGR. Lindblad et al. [7] demonstrated that the occurrence of IUGR increased with low maternal concentrations of folate and high maternal levels of homocysteine. Some studies have also reported that the incidence of IUGR during the early period of development increases when offspring do not receive sufficient nutrition from the mother [8,9]. In addition, an animal study by Burgoon et al. [6] showed that low dietary folate may lead to adverse reproductive outcomes, including an increased rate of fetal death, decreased fetal weight, and delays in palate and heart development. However, the mechanisms underlying the origin of birth defects related to folate deficiency remain largely unknown, and additional studies are needed to more precisely characterize how a lack of folate mediates disruption of the one-carbon metabolism process in abnormal embryogenesis.

Folate is a water-soluble B-vitamin (vitamin B9) that is essential for DNA and RNA synthesis and governs different methylation reactions, in which folate is a key source of the one-carbon group used to methylate DNA. Folate deficiency affects gene expression through disrupting DNA methylation patterns [10,11]. DNA methylation has been investigated in relation to malnutrition and embryonic development [12,13]. Some animal studies have provided evidence that the maternal diet during pregnancy can induce changes in DNA methylation patterns [14–16]. For example, feeding rats a low-protein and low-folic acid diet during gestation altered Igf2 and H19 expression in the livers of the offspring by regulating DNA methylation of these genes [14,15]. Feeding mice high-sugar and high-fat diets throughout pregnancy up-regulated several imprinted genes that regulate growth (Igf2, Dlk1, Snrpn, Grb10, and H19), independent of changes in DNA methylation [16]. Long interspersed nucleotide element-1 (LINE-1), a type of transposable element, accounts for approximately 19% of the genome and is regulated by methyl modifications [17]. In normal adult tissues, LINE-1 displays a silenced state with high methylation [18]. It is activated by demethylation mechanism, and then frequently

retrotransposes [19]. Folate can alter LINE-1 methylation [12]. A recent study performed by our laboratory showed that culturing mouse embryonic stem cells (ESCs) with low folate concentrations causes intracellular folate insufficiency and leads to hypomethylation of LINE-1 [20]. Another study that we performed in pregnant women with NTD fetuses demonstrated that hypomethylation of genomic DNA and LINE-1 in offspring was associated with an increased risk of NTDs [21].

Therefore, in this study we hypothesized that maternal folate deficiency can affect retrotransposition through hypomethylation, leading to IUGR. To investigate this, we established an IUGR mouse model by inducing maternal folate deficiency. We explored the potential relationship between disrupted one-carbon metabolism and the methylation levels of tissue-specific genomic DNA and LINE-1 in fetal mice with IUGR. The expression level of LINE-1 open reading frame-1 (ORF1) protein was also analyzed in IUGR fetuses. In addition, a possible correlation between global DNA and LINE-1 methylation levels and maternal serum concentrations of folic acid, 5-MeTHF, and SAM was investigated.

Material and Methods

Mice and diets

All experiments using mice were approved by the Animal Ethics Committee of the Capital Institute of Pediatrics. All efforts were made to minimize the number of mice used and the pain and discomfort that they experienced. The newly weaned C57BL/6J mice used in this study were obtained from Beijing HFK Bio-Technology Co. (JAX strains) and raised in the Institute of Laboratory Animal Science. They were fed an amino acid-defined diet based on the AIN-93G formulation [22,23], and were housed in cages that contained wire mesh flooring to prevent coprophagy. The IUGR mice model was established by feeding female mice a low-folate but isocaloric diet for 2 weeks or 4 weeks prior to mating. At 10 weeks, 120 female mice and 40 male mice were enrolled and divided into 3 groups matched for body weight. Each group of female mice was fed 1 of the following diets: a control diet (CD) containing 2 mg folate/kg; a diet deficient in folate (0 mg/kg) starting at 12 weeks (FD2w); and a diet deficient in folate (0 mg/kg) starting at 10 weeks (FD4w). All mice had free access to both food and water and were exposed to a 12: 12 h light: dark cycle in a temperature – (20–2°C) and humidity – (50±10%) controlled room. The mice were weighed every week, and the food consumption was calculated for each group. Male mice were fed the control diet.

At 14 weeks, the female mice were paired with C57BL/6J males overnight. If a sperm plug was observed on the following morning, the mice were considered to be at gestational day

(GD) 0.5. Pregnant dams received individual diets throughout gestation until the time of sacrifice. On GD13.5, all pregnant dams in the CD, FD2w, and FD4w groups were euthanized by exsanguination from the retro-orbital plexus, and blood was collected for subsequent analysis. In summary, 16 pregnant mice in each group were selected for further study. Implantation and resorption sites were counted, and placental weight, fetal weight, and embryonic crown-rump length were measured. IUGR was evaluated by the standard that the mean fetal weight in the folate-deficient group was 2 standard deviations less than that in the control group [24]. The placenta, brain, spinal cord, and liver tissues of the mouse fetuses were then harvested intact, immediately snap-frozen in liquid nitrogen, and stored at -80°C .

Serum metabolite measurements

Serum folate, 5-methyltetrahydrofolate (5-MeTHF), S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) concentrations were detected simultaneously by high-performance liquid chromatography tandem mass spectrometry (LC/MS/MS), using a method adapted from Zhang et al. [25]. A 50- μl aliquot of aqueous dithiothreitol (10 g/L) was added to 100- μl aliquots of serum, which were vortexed for 1 min and then treated with 500 μl of methanol (containing 100 mg/L each of ascorbic acid and citric acid). After the mixture was vortexed for 2 min, supernatants were obtained by centrifugation at $4000\times g$ for 15 min at 4°C and transferred to a 1.5-ml tube. The supernatants were then dried under nitrogen at room temperature and dissolved in 100- μl aliquots of methanol with a 1/3 volume of water. The Waters XevoTM TQ MS System was used for the LC/MS/MS assays. Separation was performed on a Waters UPLC BHE C18 column (2.1 \times 50 mm, 1.7 μm), and the mobile phases were held at 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B). The gradient conditions were set as follows: (flow rate, 400 $\mu\text{l}/\text{min}$): 0–1 min, 99.9% A; 1–3 min, 99.9% A to 82.0% A; 3–4 min, 82% A to 10% A; 4–5 min, 10% A; 5–6 min, 10% A to 99.9% A; and 6–6.5 min, 99.9% A. The total runtime was 6.5 min, and the injection volume was 5 μl .

DNA methylation analysis

DNA extraction

Genomic DNA was extracted from maternal peripheral blood, placental tissue, and fetal tissue using the DNeasy Blood and Tissue Kit (QIAGEN, Dusseldorf, Germany). The concentrations and quality of DNA were determined by the absorbance at 260 and 280 nm (NanoDrop 1000; Thermo Scientific, USA).

Global DNA methylation analyses

The level of global DNA methylation was determined using a new LC/MS/MS method established by our laboratory and published previously [26]. Ten nucleosides – 5-methyl-2'-deoxycytidine (5 mDC), deoxycytidine (dC), deoxyguanosine (dG), deoxyadenosine (dA), thymidine (T), 5-methyl-20-cytidine (5 mC), cytidine (C), guanosine (G), adenosine (A), and uridine (U) – were separated using an Agilent ZORBAX SB-AQ C18 column (2.1 \times 100 mm, 3.5 μm) and quantified using an Agilent 1200 LC system coupled with a tandem mass spectrometer (G6410B). The levels of methylated DNA were expressed as the ratio of 5 mDC to dG concentrations.

Bisulfite treatment

In total, 400 ng of genomic DNA from each sample was bisulfite-treated with the EZ DNA MethylationTM Kit (Eigenetics) according to the manufacturer's instructions. Sequencing analysis confirmed that >99.6% of cytosine was converted to uracil. The bisulfite-treated genomic DNA was then stored at -70°C for subsequent testing.

LINE-1 methylation analyses

The Sequenom MassARRAY platform (Sequenom, San Diego, CA) was used to perform quantitative methylation analysis of multiple CpG sites for the repetitive sequence LINE-1. The stability of this approach for quantifying methylated and unmethylated DNA has been validated by sequencing in our laboratory [21]. Bisulfite-treated DNA was PCR-amplified with T7 promoter-tagged reverse primers using an enzymatic base-specific cleavage (MassCLEAVE) process. PCR primers were designed using Methprimer (<http://epidesigner.com>). The fragment mass was then determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. LINE-1 was detected according to a published protocol established by our laboratory previously [20], and the primers (5'-aggaagagagGTTAGAGAATTTGATAGTTTTTGGAAATAGG-3' and 3'-cagtaatacgactcactatagggagaaggctCAAACAAAACCTTCTCAAACACTATAT-5') were designed to amplify a 283 base pair fragment (874–1156) in the 5'-untranslated region (5'-UTR) of LINE-1, which has internal promoter activity. A total of 10 CpG sites were examined in this area, excluding the first and seventh CpG sites, which did not fall within the limits of our detection range. Detailed information was reported in our previous study [20]. The percent of methylation at each CpG site was calculated, and a spectrum report was generated using EpiTYPER software (version 1.0, Sequenom).

Table 1. Effects of folate deficiency on fetal development indices at GD13.5*.

	CD	FD2w	FD4w
Implantations (n)	135	129	139
Resorption (%)	19 (14.07)	25 (19.38)	47 (33.81) ^{###}
Live fetuses (%)	116 (85.93)	104 (80.62)	92 (66.19)
Placental weight (g)	0.094±0.012	0.087±0.007	0.073±0.008 [#]
Crown-rump length (mm)	10.39±0.53	10.17±0.46	9.53±0.72 ^{###}
Fetal weight (g)	0.142±0.013	0.135±0.012	0.115±0.010 ^{###}
IUGR ratio (%)	7 (6.03)	14 (13.46)	64 (69.57) ^{###}

* All values are shown as the mean ±SD. CD – control diet; FD2w – folate-deficient diet for 2 weeks prior to mating; FD4w – folate-deficient diet for 4 weeks prior to mating; IUGR – intrauterine growth retardation; (n=16/group). # Significantly different from CD females, $P<0.05$; ### significantly different from FD2w females, $P<0.05$.

Table 2. Concentrations of maternal serum metabolites on GD13.5*.

	CD	FD2w	FD4w
Folate (nmol/L)	3.32±0.85	2.69±0.87	1.98±0.50 [#]
5-MeTHF (nmol/L)	46.03±8.39	23.90±7.80 [#]	8.13±5.50 ^{###}
SAM (nmol/L)	212.90±64.89	192.40±50.69	201.21±40.62
SAH (nmol/L)	286.95±88.41	420.40±99.41 [#]	710.96±113.62 ^{###}

* All values are shown as the mean ±SD. CD – control diet; FD2w – folate-deficient diet for 2 weeks prior to mating; FD4w – folate-deficient diet for 4 weeks prior to mating; 5-MeTHF – 5-methyltetrahydrofolate; SAM – S-adenosylmethionine; SAH – S-adenosylhomocysteine; (n=16/group). # Significantly different from the CD group, $P<0.05$; ### significantly different from the FD2w group, $P<0.05$.

RNA extraction and mRNA quantification

LINE-1 mRNA expression was detected by quantitative real-time PCR. Total RNA was extracted from fetal liver tissues using an RNeasy Mini Kit (QIAGEN). cDNA was synthesized from 3 µg total RNA using an oligo-dT-based reverse transcription kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Real-time PCR was carried out with a 7500 Fast Real-Time PCR System (Applied Biosystems). The following primer sequences were designed using PrimerExpress software (Applied Biosystems): ORF1p, forward 5'-GAAAGACGGCTCCAGACTAA-3' and reverse 5'-CAGACCAGGTAAGTAAAGGCAAGTT-3'; and GAPDH, forward 5'-GGCTGCCAGAACATCAT-3' and reverse 5'-CGGACACATTGGGGGTAG-3'. The PCR cycling parameters were: 50°C for 20 s, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was run in triplicate. GAPDH was used as a reference gene. The data were analyzed using the comparative CT method.

Statistical analysis

The results are expressed as the mean ±SD. The *t* test or one-way ANOVA with a Tukey test was used to examine significant

differences for pairwise or multiple comparisons, respectively. Chi-squared analysis was performed to test for a difference in proportions of variables between 2 groups. Correlation coefficients between select pairs of variables were evaluated with a two-tailed Pearson correlation test. Stepwise multiple linear regressions were performed to estimate the relationship between dependent variables (tissues methylation) and independent variables (serum metabolites). All analyses were carried out using the SPSS statistical package (version 16.0, SPSS Inc., Chicago, IL, USA), and significance was defined as a *P* value <0.05.

Results

Folate deficiency-induced IUGR mouse model and fetal development indices

We induced IUGR in a mouse model by feeding newly weaned female C57BL/6J mice with a low-folate but isocaloric diet for 2 weeks or 4 weeks prior to mating. As indicated in Table 1, the mice that received the folate-deficient diets (FD2w and FD4w groups) showed adverse reproductive outcomes, although only

Table 3. Genomic DNA methylation in maternal peripheral blood, placental tissues and fetal tissues.

	CD	FD2w	FD4w
Peripheral blood (%)	7.01±0.26	7.08±0.33	7.07±0.22
Placenta (%)	4.41±0.19	4.55±0.10	3.64±0.22 ^{#,##}
Brain (%)	7.64±0.37	7.57±0.50	7.05±0.45 [#]
Spinal cord (%)	6.41±0.49	6.04±0.28	5.67±0.49 [#]
Liver (%)	6.22±0.43	6.17±0.29	6.14±0.25

* All values are shown as the mean ±SD. CD – control diet; FD2w – folate-deficient diet for 2 weeks prior to mating; FD4w – folate-deficient diet for 4 weeks prior to mating; (n=16/group). # Significantly different from the CD group, *P*<0.05; ## significantly different from the FD2w group, *P*<0.05.

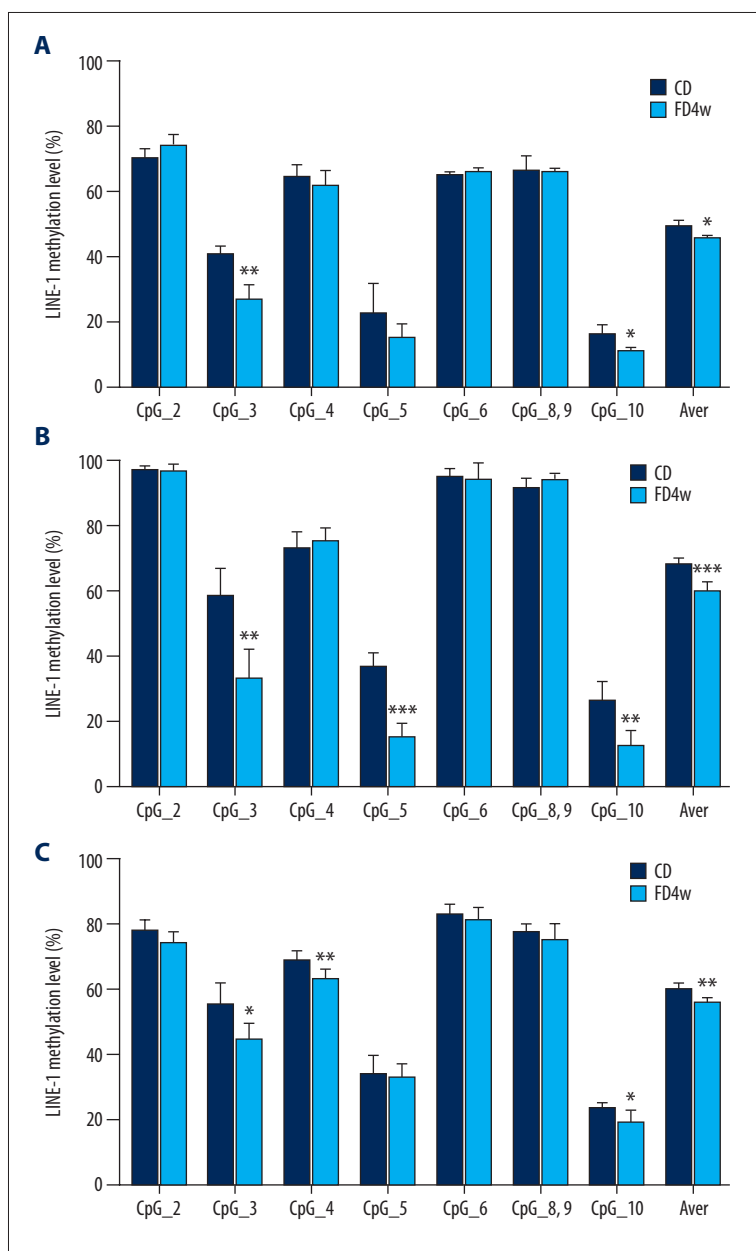


Figure 1. Methylation levels of all CpG sites in LINE-1 were compared between the CD group and the FD4w group in 3 different tissues. (A–C) LINE-1 methylation levels in placental tissue and fetal brain and liver tissues. CpG sites are numbered 1–10 from the 5' to the 3' end of LINE-1. CD, control diet; FD4w, folate-deficient diet for 4 weeks. (A) CD, n=3; FD4w, n=3. (B) CD, n=6; FD4w, n=3. (C) CD, n=5; FD4w, n=5. **** *t* test was performed: * *P*<0.05, ** *P*<0.001, *** *P*<0.0001 (relative to the respective control).

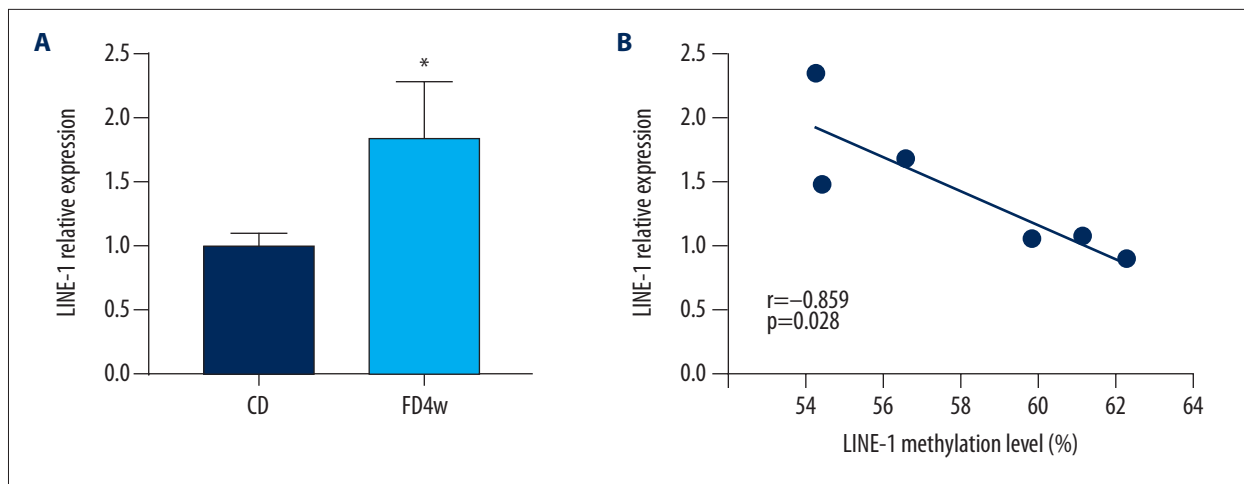


Figure 2. Increased LINE-1 ORF1p expression levels in fetal liver tissues from group FD4w. (A) ORF1p expression levels in the CD and FD4w groups. CD, control diet; FD4w, folate-deficient diet for 4 weeks. CD, $n=4$; FD4w, $n=4$. (B) Correlation between ORF1p expression and LINE-1 methylation in fetal liver tissues.

the difference between the FD4w and CD groups was statistically significant. On GD13.5, there was no statistically significant difference in the total number of fetuses, but the FD4w group had significantly higher rates of embryonic resorption than the CD mice ($P<0.001$). The average placental weight and average embryonic crown-rump length were lower in the FD4w group compared with those in the CD group ($P<0.01$ and $P<0.001$, respectively). In addition, there was a significant reduction in the mean fetal weight of the FD4w group compared with that of the control group ($P<0.001$). Consistent with the decreased fetal weights, a significant increase (11.54-fold) in IUGR incidence was observed in the FD4w group compared with the control group ($P<0.001$).

Maternal serum metabolite disturbance

Dietary folate deficiency (FD2w and FD4w diets) caused a serum one-carbon metabolism disturbance in the dams at GD13.5 (Table 2). Compared with the CD group, serum folate levels did not decrease in the FD2w group ($P>0.05$), but a marked reduction (38.9%) was observed in the FD4w group ($P<0.001$). The concentrations of serum 5-MeTHF in the dams from the FD2w and FD4w groups were 46.3% ($P<0.001$) and 81.9% ($P<0.001$) lower, respectively, than those in the CD group. However, there were no statistically significant changes found in the serum SAM concentrations among these groups ($P>0.05$). In addition, compared with the CD group, serum SAH concentrations were slightly increased in the FD2w group (1.46-fold) ($P<0.05$) and moderately elevated in the FD4w group (2.48-fold) ($P<0.001$).

Global genomic hypomethylation in various tissues

On GD13.5, the 5dmc content was measured in genomic DNA isolated from maternal peripheral blood, placental, and fetal

brain, spinal cord, and liver tissues. The 5dmc content in maternal peripheral blood from the FD2w and FD4w groups was not detectably lower than in that from the CD dietary group ($P>0.05$). However, we found that DNA from the placental and fetal tissues showed a tendency to be hypomethylated in mice that received the folate-deficient diets (FD2w and FD4w), although only some of differences between the FD4w and CD groups were statistically significant (Table 3). The 5dmc contents of placental, brain, and spinal cord tissues from the FD4w group were 17.5% ($P<0.001$), 7.7% ($P<0.05$), and 11.5% ($P<0.01$) lower, respectively, than those from the CD group, but there was no obvious decrease in 5dmc in the liver tissues between groups ($P>0.05$). These data show that the extent of DNA hypomethylation in placental and fetal tissues increase as the duration of dietary folate deficiency increases.

LINE-1 hypomethylation in placental and fetal tissues

The LINE-1 methylation levels were evaluated in maternal placental tissues and fetal brain and liver tissues in the FD4w and CD groups. In maternal placental tissues from the FD4w group, the average LINE-1 methylation level was significantly lower compared with the CD group (45.9% and 49.48%, respectively; $P<0.05$). In addition, LINE-1 methylation was also decreased in fetuses with IUGR. Compared with the CD group, the average LINE-1 methylation level in fetal brain tissue was significantly lower (60.33% compared with 68.6%, $P<0.001$). In fetal liver tissue, where folate is mainly stored, the average LINE-1 methylation level was significantly lower compared with the CD group (55.91% and 60.2%, respectively; $P<0.01$) (Figure 1).

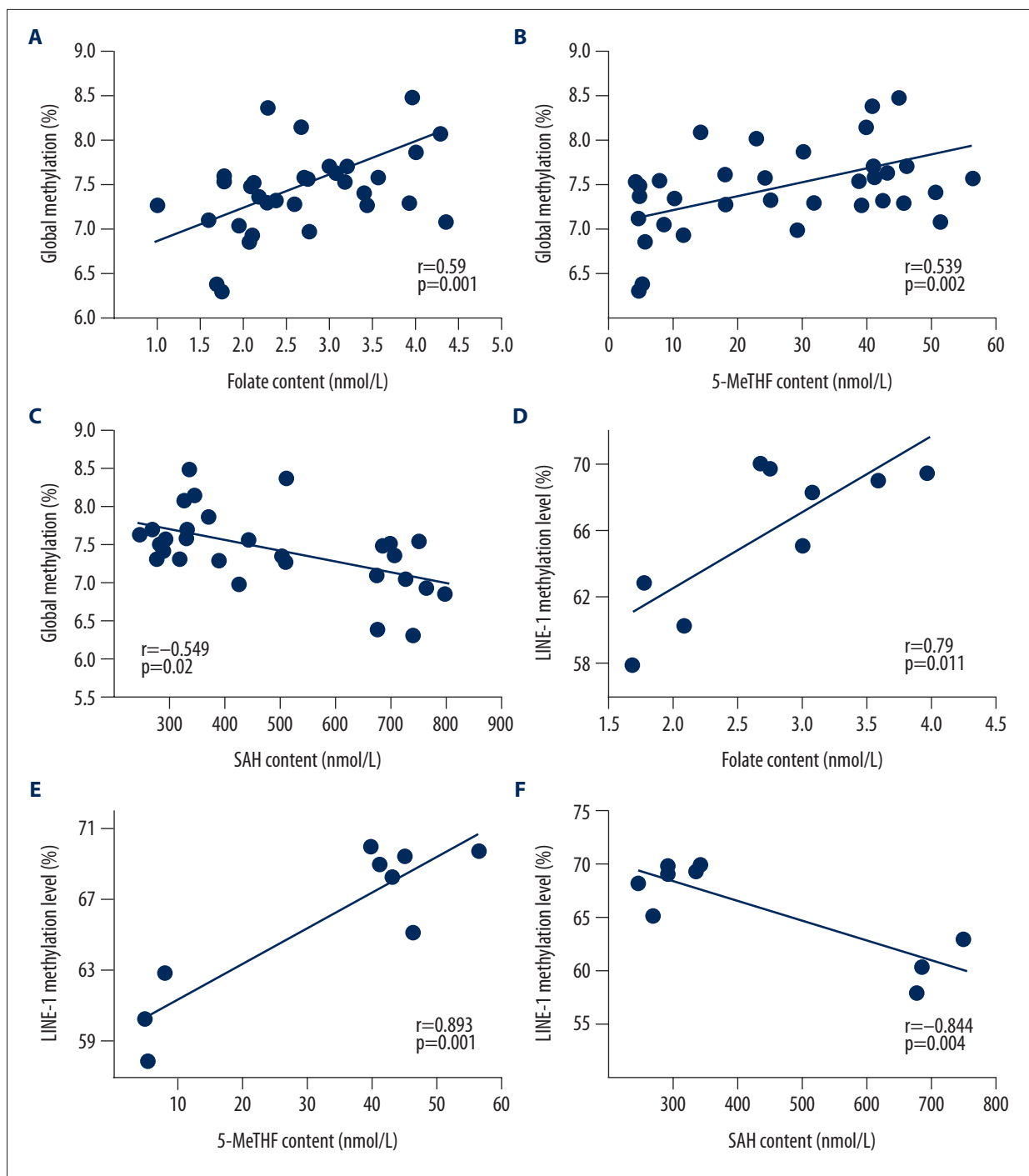


Figure 3. DNA methylation levels and one-carbon metabolites concentrations in the placenta. (A–C) Correlation between global DNA methylation and folate, MeTHF, and SAH, respectively. (D–F) Correlation between LINE-1 methylation and folate, MeTHF, and SAH, respectively. Pearson's correlation coefficient was performed.

Altered LINE-1 expression levels in fetal liver tissue

The status of ORF1p expression was measured in fetal liver tissues by real-time PCR. ORF1p expression was 2-fold higher in liver tissues from fetuses with IUGR, and this was accompanied

by LINE-1 hypomethylation (Figure 2A). Correlation analysis also showed a significant negative relationship between hypomethylation of LINE-1 and elevated ORF1p expression levels ($r=-0.859$, $P<0.05$, Figure 2B).

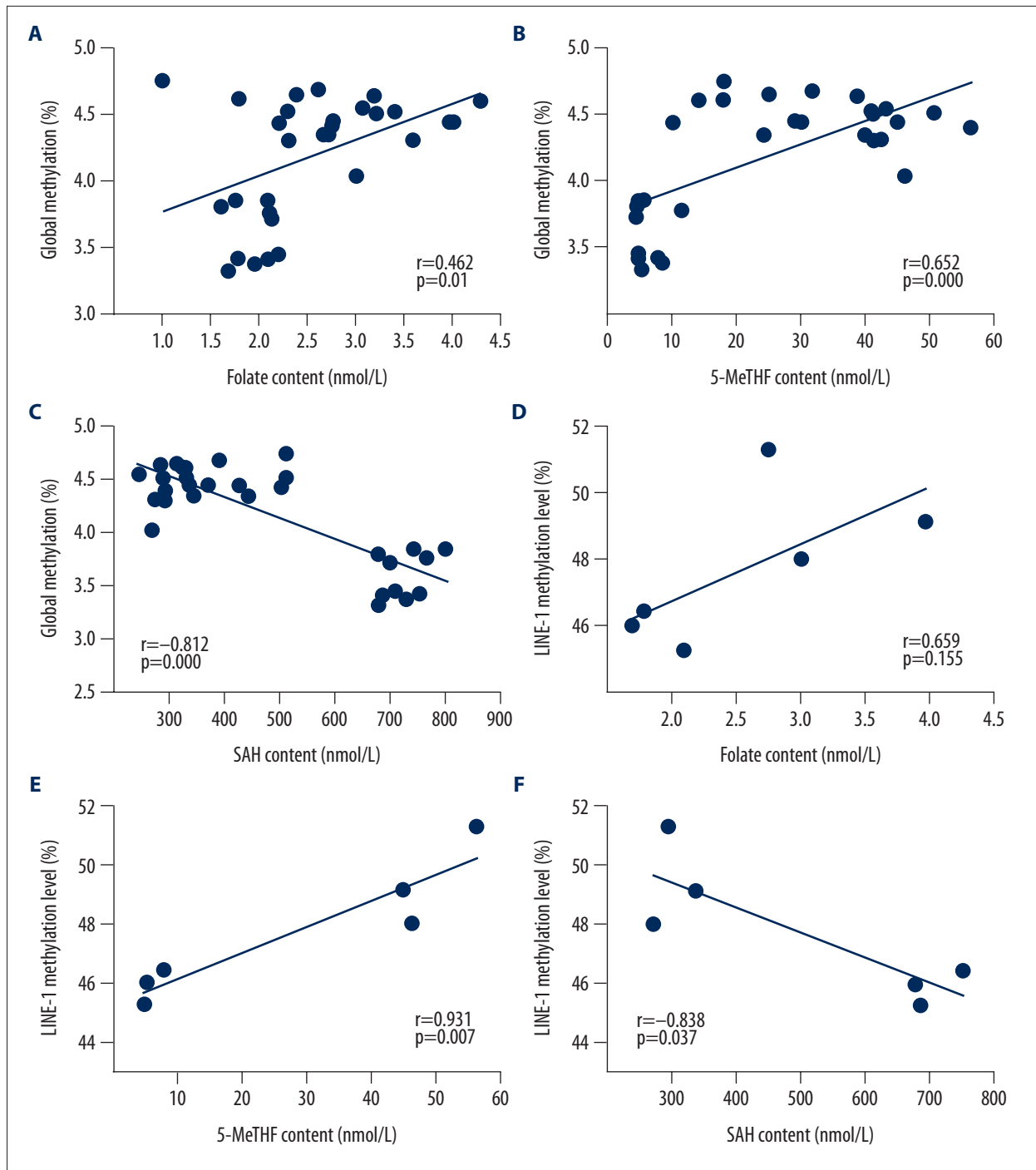


Figure 4. DNA methylation levels and one-carbon metabolite concentrations in fetal brain tissues. (A–C) Correlation between global DNA methylation and folate, MeTHF and SAH, respectively. (D–F) Correlation between LINE-1 methylation and folate, MeTHF and SAH, respectively. Pearson's correlation coefficient was performed.

Correlation between methylation levels measured in placenta and fetal tissues and maternal one-carbon metabolites

To investigate the possible relationship between maternal one-carbon metabolites and methylation status in relevant

tissues, correlation and regression analyses were performed between every serum metabolite and each tissue type. The results showed that maternal folate and 5-MeTHF content were positively correlated with genomic DNA methylation ($r=0.59$, $P<0.001$; $r=0.539$, $P<0.01$; Figure 3A, 3B), and maternal SAH

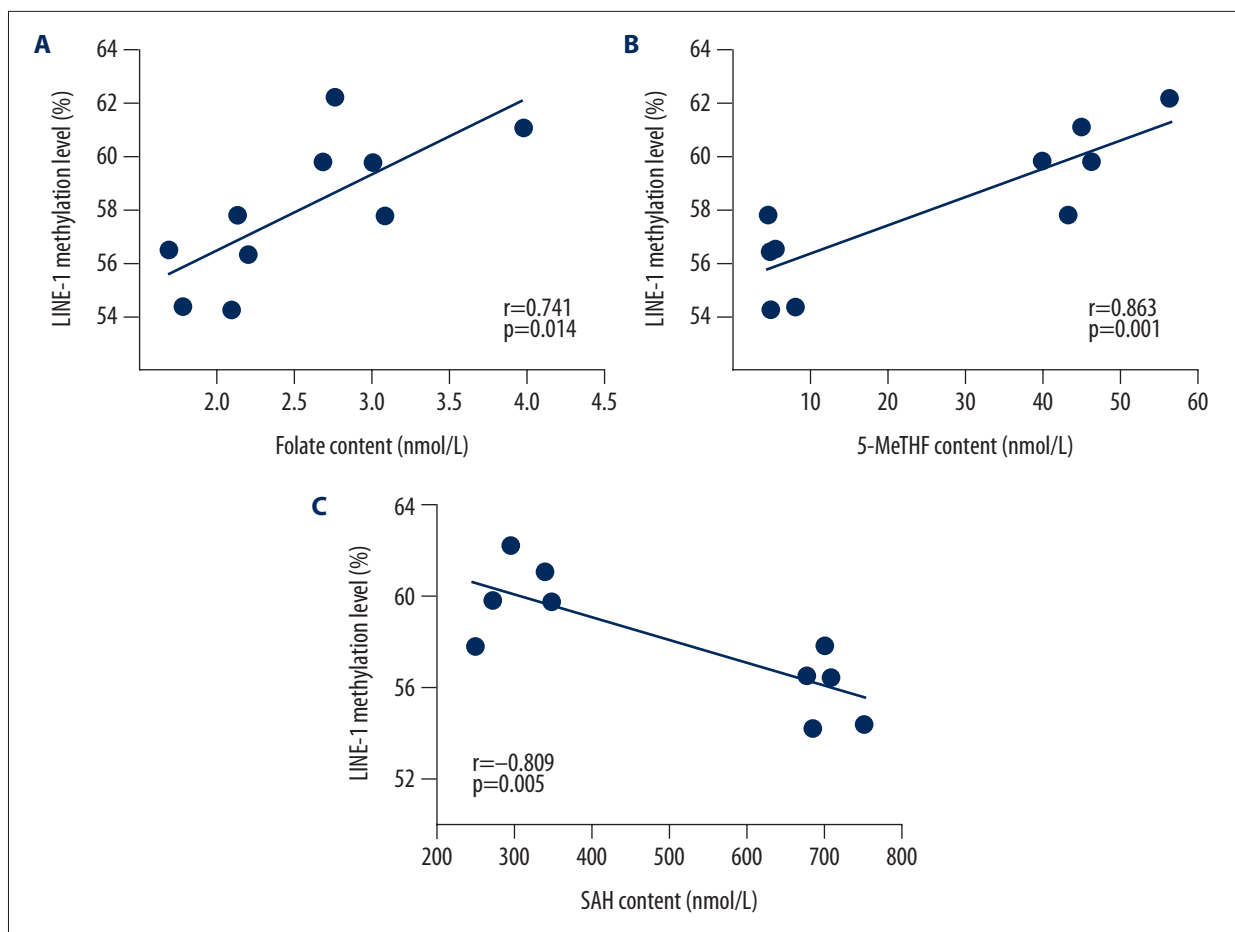


Figure 5. (A–C) LINE-1 methylation levels and one-carbon metabolite concentrations in fetal liver tissues. Pearson's correlation coefficient was performed.

content was negatively related to genomic DNA methylation ($r=-0.549$, $P<0.05$; Figure 3C) in fetal brain tissues. Furthermore, maternal folate and 5-MeTHF content were positively correlated with LINE-1 methylation ($r=0.79$, $P<0.05$ and $r=0.893$, $P<0.01$ respectively, Figure 3D, 3E), and SAH content was negatively related to LINE-1 methylation ($r=-0.844$, $P<0.01$, Figure 3F). The correlations between maternal one-carbon metabolites and global DNA and LINE-1 methylation levels in placental tissue were consistent with those in fetal brain tissues, but no significant correlation was found between maternal folate and LINE-1 methylation in placental tissue (Figure 4). In fetal liver tissue, maternal folate and 5-MeTHF concentrations were positively correlated with LINE-1 methylation, and SAH concentrations were negatively correlated with LINE-1 methylation (Figure 5). We observed the same trend between metabolite levels and global DNA methylation in the maternal spinal cord (Supplementary Figure 1).

Discussion

In this study, we established an IUGR mouse model by feeding pregnant dams a folate-deficient diet and identified genomic DNA and LINE-1 methylation alternations in the fetuses related to IUGR. As our results show, a maternal diet deficient in folate led to disruption of one-carbon metabolism in the maternal plasma in a mouse model of IUGR, including decreased folate and 5-MeTHF levels, as well as an increase in SAH. At the same time, global DNA and LINE-1 methylation levels were disturbed in fetuses with IUGR. As a result, ORF1p was expressed at higher levels in fetuses with IUGR.

Folate deficiency during pregnancy has been linked to developmental abnormalities such as preterm delivery, low-birth-weight infants, NTDs [27–29], and cleft palates [30]. We previously showed that treating mice with 5-FU, RTX, or MTX, which disrupt folate metabolism via different mechanisms, caused a similar spectrum of embryonic malformations, such as growth retardation, embryonic resorption, NTDs, and other malformations [31–33]. Therefore, in this study, we measured

related development indices in fetal mice. We found that maternal consumption of a folate-deficient diet before pregnancy increased the rate of adverse fetal outcomes, including higher rates of embryonic resorption and lower placental weight.

Different durations of dietary folate deficiency were explored to determine the cumulative effect of folate. Data from maternal plasma suggested that only long-term folate deficiency induced IUGR. The fetal weights in the FD4w group only were significantly lower compared with the control group, which led to a distinct increase in the incidence of IUGR. However, fetal weight only decreased slightly compared in the FD2w group compared with the CD group, and this diet did not cause any notable growth retardation in the fetuses. Folic acid metabolism in the maternal plasma confirmed the significant decrease in folate in the FD4w group compared with the FD2w group. We further observed that the levels of the folate metabolites 5-MeTHF and SAH were more significantly altered in the FD4w group than in the FD2w group. In a folate deficiency study in C57BL/6N mice [34], significantly lower weight gain was observed for folate-deficient mice after 10 weeks. In another folate deficiency study in rats, a significant difference was observed even after 3 weeks [35]. Combined with these studies, our findings allow us to speculate that long-term folate deficiency results in deficient accumulation, leading to embryonic development abnormalities.

In our study, we found that placental and fetal genomic DNA from mice in the FD4w group was significantly hypomethylated. LINE-1 methylation levels were evaluated in placental tissue and fetal brain and liver tissues, all of which are closely related to fetal growth. Given that only the FD4w diet led to IUGR, these tissues were selected from the FD4w group. Notably, we found significant LINE-1 hypomethylation in tissues from fetuses with IUGR. Results from our previous study also indicated that LINE-1 Homo sapiens (L1Hs) hypomethylation increases the risk of NTDs [36].

LINE-1 elements contain 2 open reading frames (ORF1 and ORF2) that encode 2 proteins: LINE-1 ORF1p and LINE-1 ORF2p [37]. ORF1p has RNA binding activity [38], and ORF2p acts as a reverse transcriptase [39] and an endonuclease [40]. In our study, the decrease in LINE-1 methylation was associated with a 2-fold increase in L1 ORF1p expression in liver tissues of fetuses with IUGR, suggesting that retrotransposition was significantly increased in these tissues. Chromosome stability is essential for embryonic development, and structural chromosomal abnormalities can induce various mutations [41]. Our previous study suggested that disrupted folate metabolism by treatment with MTX is associated with a higher proportion of CNVs in NTDs, in which both chromosome malsegregation and breakage increased in cases [42]. Our data show that overexpression of LINE-1 ORF1p and LINE-1 hypomethylation may be due to increased retrotransposition within the genome, leading to genome instability and, eventually, IUGR.

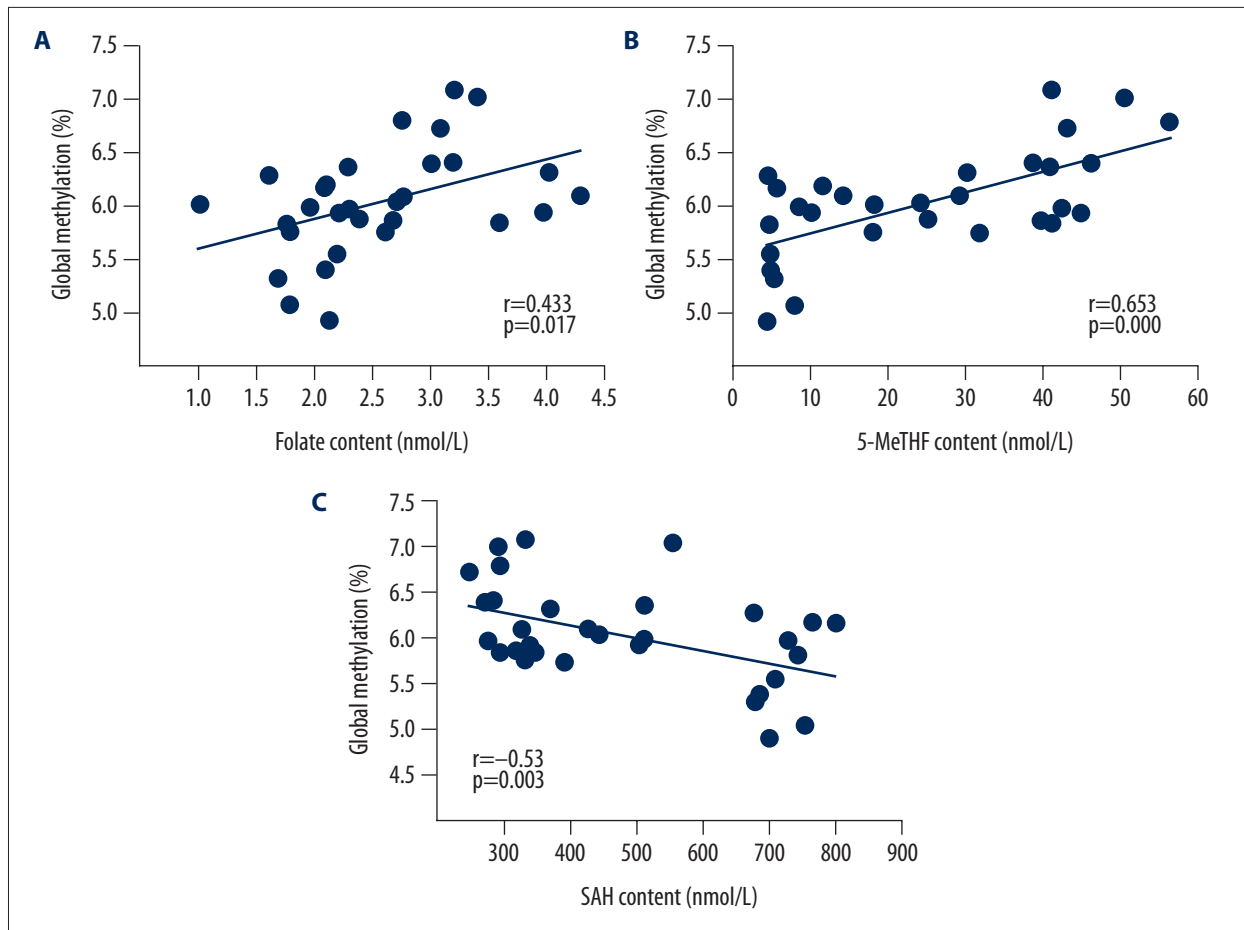
Conclusions

In conclusion, our study found that disordered one-carbon metabolism caused by maternal folate deficiency leads to an increased risk of mice IUGR by reducing global and LINE-1 methylation levels, which was correlated with higher expression levels of ORF1p. Our data suggest that improvements in nutrition during early pregnancy can prevent IUGR, possibly via the donation of methyl groups that are important for embryo development. Further research in human populations is warranted.

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Supplementary Figure



Supplementary Figure 1. (A–C) Global DNA methylation levels and concentrations of one-carbon metabolites in maternal spinal cord tissue. Pearson's correlation coefficient was performed.

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