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Tissue-specific distribution of aberrant DNA methylation associated with maternal low-folate status in human neural tube defects☆

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Abstract

This study compares the density and tissue-specific distribution of 5-methyl cytosine (5mC) in genomic DNA from human fetuses with or without neural tube defects (NTD) and examines whether low maternal serum folate is a possible correlate and/or risk factor for NTD. The results demonstrate significant hypomethylation of brain genomic DNA in NTD fetuses relative to controls ($P<.01$), as well as relative hypermethylation of skin and heart in NTD fetuses. In normal fetuses, the level of 5mC in liver genomic DNA decreased from fetal week 18 to 28 and increased over the same developmental period in kidney genomic DNA, but these trends were absent in genomic DNA from NTD fetuses. Mean maternal serum folate was significantly lower in NTD fetuses than in controls ($P<.01$), and maternal serum folate correlated with density of 5mC in genomic brain DNA from NTD fetuses ($r=0.610$). The results indicate that aberrant DNA methylation in NTD may be due to maternal folate deficiency and may be involved in the pathogenesis of NTD in humans.

Keywords: Hypomethylation; Neural tube defect; Tissue-specific methylation; Folate deficiency; Human fetus

1. Introduction

Neural tube defects (NTD) are severe congenital malformations in which the embryonic neural tube fails to form correctly during development. The worldwide incidence of NTD is 1 per 1000 live births, with significant regional variation, such as 14 NTD cases per 1000 live births reported in 2003 in Northern China [1]. Maternal folate intake/deficiency is thought to be a critical environmental risk factor for NTD. Significantly, maternal periconceptional supplementation with folic acid reduces the incidence of NTD [2,3]. The precise mechanism by which dietary folate might prevent NTD is not well understood, but because biological derivatives of folate are critical for interlinked one-carbon (i.e., methyl donor) metabolism, it has been suggested that low folate-induced aberrant patterns of methylation could play a causal role in NTD [4,5]. In particular, 5-methyltetrahydrofolate is required for re-methylation of homocysteine to methionine, which is a precursor of *S*-adenosylmethionine (SAM). SAM is the principal methyl donor in many biochemical reactions, including the methylation of cytosine in DNA. Human and animal studies suggest that folate status influence global patterns of DNA methylation [6,7]. Consistent with this, suppression of one-carbon metabolism or insufficient methionine intake, increase the incidence of NTD [8,9]. Reduced DNA methylation is also associated with incomplete closure of the cephalic neural tube in rat embryos in vitro [10]. Thus, folate deficiency may increase the risk of NTD by decreasing DNA methylation.

DNA methylation is a major epigenetic modification of the genome that plays a critical role in transcriptional regulation, X-chromosome inactivation, genomic imprinting, tissue-specific gene expression, and chromosomal stability in eukaryotes [11]. It is also essential for embryogenesis and proper fetal development [12]. DNA methylation profiles in mammals are tissue-specific [13,14]. Genomic methylation patterns are erased in the early embryo, re-established in a cell- or tissue-specific pattern as embryogenesis proceeds, and remain largely unchanged in adult cells. Mammalian DNA methyltransferases (Dnmts) catalyze methylation in genomic DNA using SAM as methyl donor [15]. Elimination of Dnmt1, Dnmt3a or Dnmt3b reduces global

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DNA methylation in embryos and causes embryonic lethality in mice [16]. Aberrant DNA methylation is associated with several human congenital diseases, including Beckwith–Wiedemann and Prader–Willi/Angelman syndromes [17,18]. Animal studies have established a link between aberrant DNA methylation and NTD [10,19], and the level of DNA methylation in mammalian genomic DNA is regulated in a tissue–specific manner. However, the relationship between aberrant tissue–specific DNA methylation and NTD in humans has not been rigorously examined.

This goal of this study was to investigate DNA methylation status in multiple tissues of second trimester NTD and control human fetuses, and explore the possible correlation between aberrant DNA methylation and maternal folate status. Quantitative high-performance liquid chromatography (HPLC) was used to measure 5-methylcytosine (5mC) in genomic DNA from fetal tissues from three germ layers of second gestation period human NTD and control fetuses. The density and tissue-specific distribution of 5mC was compared and correlated with maternal folate status for NTD and control fetuses. This work contributes to our understanding of the role of DNA methylation in human development, possible molecular mechanisms involved in NTD, and the importance of folate status as a risk factor for NTD.

2. Materials and methods

2.1. Human tissue and blood samples

Samples were obtained from hospitals in Lvliang District, Shanxi Province, Northern China, where the NTD prevalence was reported to be 199.38/10 000 based on local epidemiological surveillance data from January 2002 to December 2004 [20]. This study was approved by the Committee of Medical Ethics of the Capital Institute of Pediatrics, Beijing, P. R. China.

NTD tissue samples were collected with the informed consent of the parents between 18–28 gestational weeks after pregnancy termination by mid-term induction. Pregnancy termination in such cases was done after determining that there is a fetal NTD abnormality (spina bifida, encephalocele or anencephaly) via ultrasound diagnosis by a fetal pathologist. Normal tissues were obtained from abortions (18–28 gestational weeks) after receiving the informed written consent of families or relatives at the hospital. The tissue samples were immediately frozen and stored at -80° C.

Maternal blood samples were obtained from case and control pregnancies within one week of obtaining consent. Venous blood samples were collected into red-top Vacutainer tubes (without anticoagulant; Becton Dickinson) by the trained staff from a local hospital. These were immediately centrifuged at 2500 rpm for 10 min, after which serum was separated and stored without a reducing agent at −20°C in local hospitals until samples were shipped (on ice) to laboratories. The samples were not allowed to thaw until immediately before analysis.

2.2. DNA preparation and digestion

Genomic DNA was extracted from brain, skin, heart, kidney, lung and liver using a Nucleon DNA Extraction Kit according to the manufacturer's recommendations. Around 50 mg of tissue was used for DNA extraction per column, and elution was done with a 50–110 μl TE buffer.

Approximately 50 μg DNA was dissolved in 300 μl of 1×TE. RNaseA was added to a final concentration of 100 μg/ml, followed by RNase T1 to a final concentration of 2000 units/ml. The solution was then incubated at 37°C for 2 h, followed by ethanol precipitation. After RNA removal, DNA treatment with DNase I and Nuclease P1 (Sigma-Aldrich, St. Louis, MO, USA) was performed as described by Mills et al [21].

2.3. Analysis of DNA methylation by HPLC

Deoxycytidine 5′-monophosphate (C), 5-methyl-2′-deoxycytidine 5′-monophosphate (5mC), 2′-deoxyguanosine 5′-monophosphate (G), deoxyadenosine 5′-monophosphate (A), and thymidine 5′-monophosphate (T) were purchased from USB (Cleveland, OH, USA). 5mC, C, T, A and G were separated by high-performance liquid chromatography (HPLC) using an ESA C18 column (250 mm×3.2 mm I.D., 3μm particle size, Chelmsford, MA, USA) using 50 mM ammonium orthophosphate (pH 4.1) as mobile phase and detected by absorbance spectroscopy at 278 nm at a sensitivity of 0.005 absorbance-unit full scale. All DNA samples were analyzed in duplicate and experimental samples were compared to profiles of standard compounds. The difference in 5mC content between duplicate samples was less than 3%. The method was validated by determining the linearity ($r^2 \geq 0.999$), and the coefficients of variation of intra- and inter-day precision were less than 5%. To minimize experimental error between groups, six tissue samples from one patient fetus and the matched control, together with a control DNA from the peripheral blood of a healthy individual, were analyzed during each experiment. All experiments were performed blind (i.e., specimens were treated as unknowns). %5mC was calculated as the proportion of total 2′-deoxycytidine-5′-monophosphate (5mC+C) using the following equation: $\%5mC = [(5mC)/(5mC + C)] \times 100$.

2.4. Folate status analysis

Serum folate was measured by competitive receptor binding immunoassay (Chemiluminescent Immunoenzyme Assay Access Immunoassay system; Beckman Coulter, Krefeld, Germany) [22]. The intra-assay CV for serum folate was less than 6.2%. All sample analyses were performed blind.

2.5. Statistical analysis

Statistical analyses were performed using SPSS version 10 for Windows 2000. Statistical significance of differences in continuous variables was evaluated by Student's t-test or one-way analysis of variance (ANOVA) for more than two groups. Values with a $P<.05$ (two-tailed analysis) were considered statistically significant. Correlations were evaluated using a two-tailed Pearson correlation test, and were considered statistically significant at $P<.05$.

3. Results

3.1. Altered tissue-specific distribution of 5mC in NTD fetuses

Tissue-specific DNA methylation plays an important role during the embryonic stages of mammalian development. Because it has been proposed that aberrant one-carbon metabolism and altered patterns of DNA methylation could play a role in NTD, we measured the density of 5-methylcytosine (5mC) in genomic DNA from brain and skin (ectodermic-derived), heart and kidney (mesoderm-derived) and liver and lung (endoderm-derived) in 20 NTD (18 spina bifida tissues, 2 anencephalus residual tissues) and 20 age- and sex-matched control human fetuses. 5mC content was determined by HPLC and expressed as %5mC {i.e., $[(5mC)/(5mC + C)]\times100$}. A specific 5mC data in NTD brain was picked out.

In the skin, heart, lung, brain, kidney and liver tissues from the control fetuses, mean 5mC content increased sequentially (Table 1). Mean 5mC content was lower in DNA from skin, heart, and lung than in DNA from brain, kidney and liver. However, the distribution of DNA methylation in all types of tissues was altered in the NTD samples. Mean 5mC content in brain from NTD fetuses was lower than 5mC content in other tissues from NTD fetuses.

Analysis of the distribution differences by one-way ANOVA revealed that the distribution of methylation between tissues from the NTD samples and the control group was significantly different ($P=.001$). The least significant difference (LSD) method was then used to make independent pairwise comparisons (Table 2). In control samples the largest differences in %5mC were heart vs. liver and skin

Table 1
Mean %5mC in endo-, meso- and ectodermic tissues from NTD and control human fetuses

Group	Heart (20)	Skin (20)	Lung (20)	Brain (19)	Kidney (20)	Liver (20)
NTD	3.512 (0.100)	3.585 (0.088)	3.501 (0.080)	3.410 (0.078) *	3.755 (0.141)	3.874 (0.094)
Control	3.347 (0.087)	3.393 (0.075)	3.542 (0.105)	3.650 (0.071)	3.668 (0.106)	3.858 (0.087)

Values are means %5mC±(S.E.M).
* $P<.01$ (compared with control).

Table 2
P values for pairwise comparison of %5mC in tissues from NTD or control human fetuses

Group	h-li	h-lu	h-k	h-s	h-b	li-lu	li-k	li-s	li-b	lu-k	lu-s	lu-b	k-s	k-b	s-b
NTD	0.011 *	0.93	0.086	0.603	0.472	0.009 **	0.396	0.042 *	0.001 **	0.073	0.546	0.524	0.230	0.017 *	0.219
Control	0.000 ***	0.125	0.012 *	0.717	0.027 *	0.014 *	0.316	0.000 ***	0.0740	0.319	0.241	0.485	0.031 *	0.764	0.063

h, heart; li, liver; lu, lung; k, kidney; s, skin; b, brain.
* $P<.05$.
** $P<.01$.
*** $P<.001$.

vs. liver, ($P<.001$), and somewhat smaller differences were observed for heart vs. kidney, heart vs. brain, lung vs. liver, skin vs. kidney ($P<.05$). Interestingly, the relative 5mC content in different tissues was significantly different in NTD fetuses than in control fetuses. In particular, 5mC content was not significantly different for heart vs. brain or kidney, or skin vs. kidney, while significant differences were observed for brain vs. liver ($P<.001$) and brain vs. kidney ($P<.05$). These results demonstrate that the tissue-specific distribution of 5mC in an NTD fetus is significantly different from the tissue-specific distribution of 5mC in a normal (non-NTD) fetus.

Mean 5mC differences in one tissue type between the NTD samples and the matched controls (Mean $\%5mC_{NTD}$ − Mean $\%5mC_{control}$) were compared with those of another tissue by a paired-sample t-test (Fig. 1). In brain, skin, heart, kidney, liver and lung tissues, the absolute value of mean 5mC difference between cases and controls increased sequentially. The mean difference in 5mC between cases and controls was significantly different between the heart, skin, or kidney, and the brain. This implies that the alteration in DNA methylation in brain tissues is more significant and that DNA of skin and heart tissues has a tendency to be hypermethylated in NTD.

3.2. Global DNA hypomethylation of brain tissue in NTD

To examine the extent of changes in DNA methylation in each tissue type, the difference in 5mC level between the NTD and the control groups was analyzed for each tissue type. The greatest difference in 5mC content was found in brain tissue ($P=.009$, Table 1), with the NTD group displaying hypomethylation. There was no significant difference in the 5mC content of skin ($P=.057$) or heart ($P=.10$) tissues, nor of liver, lung and kidney tissues between the NTD and the control groups. These imply that brain tissue is the target of hypomethylation in NTD.

To better understand this difference, 25 additional NTD brain samples, including five encephalocele samples, 10 spina bifida samples and 10 anencephalus samples, were included in the survey. These were matched with 25 control brain samples. The difference between the expanded NTD ($n=44$) and the control ($n=45$) groups was still significant (3.190%±0.081% vs. 3.566%±0.074%, $P<.001$). There were no significant differences between the anencephalus and

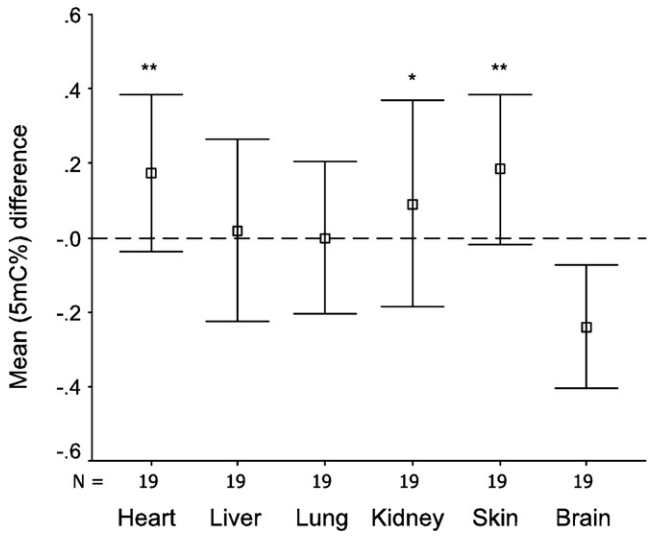

Fig. 1. Comparison of relative 5mC content among different tissues. (The *y*-axis represents relative 5mC content [Mean $\%5mC_{NTD}$–Mean $\%5mC_{control}$] and the S.E.M.). **P*<.05 (comparison of relative 5mC content difference between the kidney and the brain); ***P*<.01 (comparison of relative 5mC content difference between the heart or skin and the brain).

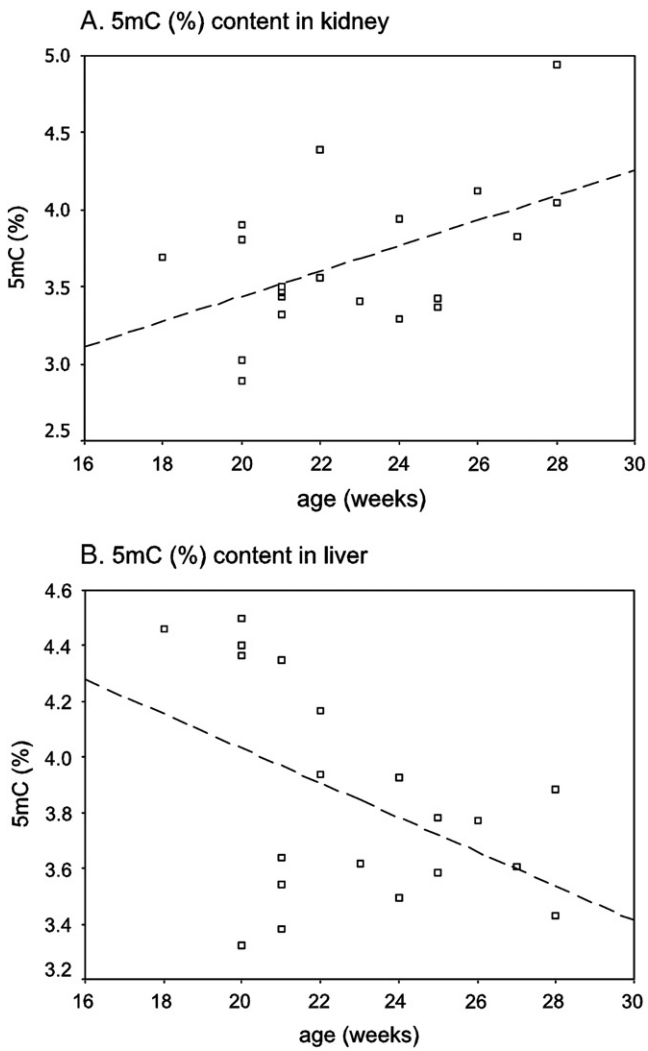

Fig. 2. 5mC content of genomic kidney and liver DNA from 18 to 28 weeks old normal human fetuses. (A) Kidney. (B) Liver.

Table 3
Maternal serum folate in women pregnant with NTD or control fetuses

	NTD (n=20)	Control (n=20)
Folate (nmol/L)	8.930	12.858
Natural log (LN) folate Mean (S.E.M)	2.138 (0.076) *	2.470 (0.093)

* P<.01 (compared with control).

the spina bifida groups, between the spina bifida and the encephalocele groups, or between the anencephalus and the encephalocele groups (data not shown).

3.3. Age-associated change in 5mC content in liver and kidney during normal but not NTD fetal development

Previous studies demonstrated that patterns of DNA methylation change in a tissue-specific manner during embryonic stages of rat development [23]. This result was confirmed in this study using liver and kidney DNA from human fetuses (Fig. 2). Interestingly, 5mC content increased in kidney DNA (r=0.504, P=.038) and decreased in liver DNA (r=−0.467, P=.036) from Week 18 to 28 of embryogenesis. In contrast, 5mC content in other tissues from control fetuses and all tissues from NTD fetuses did not correlate with or appear to be significantly influenced by embryo age during the same developmental period (P>.05). The influence of gender on 5mC content in NTD and control fetuses was not investigated in this study.

3.4. Maternal serum folate concentration and fetal DNA methylation

Folate deficiency compromises one-carbon metabolism and decreases capacity for DNA methylation and is known risk factor for NTD. Therefore, maternal serum folate during pregnancy was quantified using a competitive receptor binding immunoassay. Folate concentration was not distributed normally, so it was transformed to a normal distribution via a transform power function of SPSS and tested using Student's t test. Table 3 shows that the mean serum folate concentration was significantly lower in pregnant mothers carrying NTD fetuses than in pregnant mothers carrying control fetuses (P<.01).

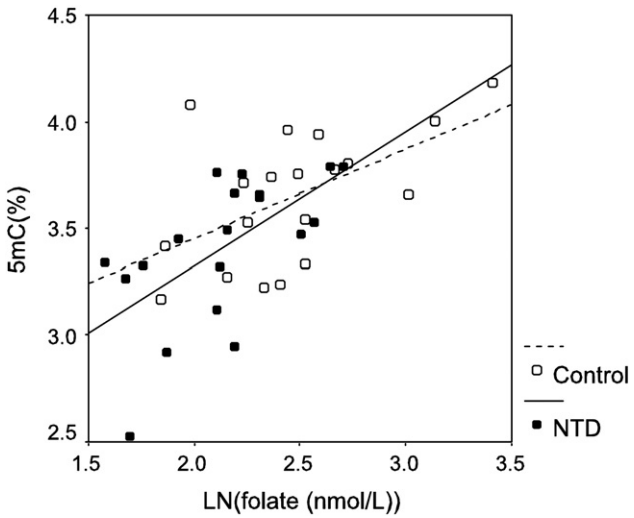

Fig. 3. Maternal serum folate against 5mC content in genomic DNA from NTD and normal human fetal brain.

The relationship between 5mC content and maternal serum folate was also examined in brain and other tissues of individual NTD and control fetuses. Mean 5mC content was lower in brain DNA of NTD fetuses than in brain DNA of control fetuses (Fig. 1, Table 1); however, in addition, 5mC content in brain DNA from individual NTD fetuses was significantly correlated with maternal folate concentration (log-transformed) during pregnancy (r=0.610, P<.01). The same correlation was observed in DNA from normal fetal brain (r=0.535, P<.05, Fig. 3). However, 5mC content of other NTD and control fetal tissues was not significantly correlated with maternal folate concentration in this study.

4. Discussion

This study is the first analysis of tissue-specific DNA methylation in human NTD fetuses. The main conclusions of the study are: (1) the distribution of 5mC in NTD fetuses is significantly different from the distribution of 5mC in control fetuses; (2) the alteration of DNA methylation in brain tissue is more significant than in other tissues; (3) 5mC content in brain from NTD fetuses is significantly lower than 5mC content in brain from control fetuses; (4) mean maternal folate concentration is significantly lower in NTD fetuses than in control fetuses and (5) maternal serum folate concentration correlates with 5mC content in NTD brain. These observations support the idea that folate deficiency during human pregnancy leads to aberrant patterns of DNA methylation in fetal DNA during fetal development. This could explain the increased incidence of NTD-affected pregnancies in folate-deficient women. Folate deficiency and associated DNA hypomethylation are also associated with increased cancer risk, suggesting that DNA hypomethylation may play a role in other pathological processes during or after embryonic development [24,25].

Our results show that the distribution of 5mC in NTD fetuses is different from the distribution of 5mC in control fetuses (Fig. 1, Tables 1 and 2), with the most marked observation being hypomethylation in the brain (relative to other NTD tissues and to brain in control fetuses) and hypermethylation in the skin and heart in NTD fetuses relative to control fetuses. Based on this observation, we hypothesize that aberrant DNA methylation leads to altered gene expression during embryonic development [26], which in turn causes development defects including malformation of fetal neural structures. For example, hypomethylation in cells in the neural lineage could lead to aberrant expression of one or more genes that are essential for cranial neurulation. Consistent with this idea, disruption of Dnmt 3b during embryonic development causes cranial NTD [27].

In normal human fetuses, we observed tissue-specific hypermethylation (liver, kidney and brain) and age-specific increase (kidney) or decrease (liver) in DNA methylation, but no age-specific changes in lung, brain or heart DNA methylation. These results differ from the results of previous studies [13,23]. One previous study of tissue-specific DNA methylation was limited to ten matched pairs of samples from adults of different ages [13]; in contrast, in the present study, various tissues from the same fetus were compared, and the number of sample pairs was 20. The larger sample size in this study provides increased statistical power, possibly allowing detection of significant differences within the current data set that could not be confirmed in the smaller study conducted previously. In addition, a previous study showed age-associated change in methylation of rat fetal brain and heart DNA, but no age-associated change in rat fetal lung DNA [23]. This could indicate a species-specific difference.

Interestingly, although 5mC content of kidney and liver DNA are similar in NTD and control fetuses, age-related changes in kidney and liver DNA methylation were only observed in control fetuses. This suggests aberrant regulation of DNA methylation in kidney and liver in NTD fetuses. Hypermethylation of liver in older NTD fetuses might cause aberrant gene expression, as discussed above, leading to altered

metabolism or developmental defects. Hypomethylation in kidney in older NTD fetuses might have a similar effect, leading to kidney-related defects such as absence or minification of one kidney, which have been observed in NTD fetuses [28].

Several limitations of the current study should be pointed out. By the end of the first pregnancy trimester, many human fetal tissues are fully formed and stable patterns of DNA methylation are achieved in these tissues. In addition, closure of the neural tube is normally completed within the first 28 days following conception of the human fetus. However, this study was conducted using DNA from 18- to 28-week old fetuses. This is a limitation, because additional age- and tissue-specific patterns of DNA methylation might be detected in samples from earlier stage embryos/fetuses, if they were available and could be included in the analysis. Unfortunately, tissue samples, especially brain tissue, from fetuses of <28 days old are difficult to obtain. Another limitation of the present study is that the levels of metabolites, such as folate, SAM and methionine, in fetal tissues could not be determined and were not included in the analysis.

The findings presented here raise two fundamental and related questions. First, why is brain DNA in NTD fetuses hypomethylated? The data in this study show that mean maternal folate concentration is significantly lower in NTD fetuses than in control fetuses and that maternal serum folate concentration correlates with 5mC content in NTD brain. Thus, it is reasonable to propose that folate deficiency interferes with normal one-carbon metabolism and causes an overall decrease in DNA methylation in the developing fetus. Specifically, we propose that DNA hypomethylation in NTD brain tissue is a passive phenomenon, resulting from decreased supply of methyl-donor groups (i.e., low level of SAM). Alternatively, it is possible that defective expression or alternative splicing of one of the DNA methyltransferases, Dnmt 1, Dnmt 3a or Dnmt 3b, plays a role in hypomethylation of brain DNA in NTD fetuses.

Second, why and how are some tissues in the developing NTD fetus hypomethylated, while other tissues are hypermethylated? One possible explanation is that some but not all fetal tissues can activate an alternative pathway that compensates for low levels of folate-derived methyl donor groups. In particular, in some tissues, homocysteine can be converted to methionine by betaine-homocysteine methyltransferase, using betaine as a methyl group donor. Betaine-homocysteine methyltransferase is primarily expressed in the liver and kidney [29]. This is consistent with the observations that the level of DNA methylation is close to normal in liver and kidney of NTD fetuses in this study, and in liver from rats under moderate folate deprivation in previous studies [24,30]. Another possible explanation is that the tissue-specific effect of folate deficiency reflects tissue-specific and developmentally-regulated expression of Dnmts during embryonic development. For example, Dnmt1 is expressed more highly in brain than in other tissues [31]. We propose that compensatory up-regulation of Dnmt may cause DNA hypermethylation in the heart and skin of NTD fetuses. However, because Dnmt1 is already expressed at a high level in the brain, it may not be possible to raise Dnmt activity further, to compensate for reduced methyl donor capacity. Similarly, reduced or defective Dnmt1 activity may have a larger effect in brain, causing greater reduction in 5mC content and higher degree of chromosomal instability in the brain than in other tissues in the NTD fetus. Further investigation of alternative splicing pathways for of each of the Dnmts is needed to test this hypothesis.

The possible importance of tissue-specific aberrant DNA methylation, especially hypomethylation of fetal brain, as a causal factor in NTD has been demonstrated in this study. However, the precise mechanism by which hypomethylation in brain might cause NTD is not yet known and requires further study. Such future research could include genome-wide analysis of CpG island methylation or studies of proposed gene-specific effects that have functional consequences during neural development. The current study and future follow-up studies will likely make a significant contribution to our understanding of the pathological consequences of altered DNA methylation during human fetal development.

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