Maternal high-methyl diet suppresses mammary carcinogenesis in female rat offspring

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Maternal nutrition during pregnancy influences the development and metabolism of the fetus. Recent studies suggest that the cancer risk of offspring later in life is associated with maternal diet, but little is known about the effect of a maternal diet high in methyl nutrients on breast cancer risk. Lipotropes are methyl group-containing essential nutrients (methionine, choline, folate and vitamin B\textsubscript{12}) that play key roles in one-carbon metabolism. In this study, we investigated the long-term effects of maternal dietary high-dose lipotropes (five times higher than in the control diet) on the development and progression of mammary tumors in rat offspring using two separate experiments (in utero exposure with and without postnatal supplementation). In both experiments, the female offspring were injected intraperitoneally with a single dose (50 mg/kg body wt) of N-nitroso-N-methylurea during puberty to induce mammary tumors. Tumor growth and development were recorded, and at the end of the study, tissues were collected for analysis. For both experiments, the offspring from dams fed a high-dose lipotrope showed significantly decreased tumor incidence, tumor multiplicity and tumor volume, while also displaying a significant increase in survival rate and tumor latency. Gene transcription analysis, as measured by quantitative real-time PCR, revealed a significant decrease of histone deacetylase 1 (Hdac1) messenger RNA in mammary tumors in both experiments. Our findings provide evidence that maternal dietary high-dose lipotropes reduce mammary carcinogenesis in offspring in association with long-term alterations in gene expression and may be useful in developing maternal dietary strategies to prevent breast cancer.

Introduction

Maternal nutrition is known to affect the intrauterine environment and thereby plays a critical role in fetal development and metabolism (1). Recent studies indicate that some risk factors in adult chronic diseases such as cancer and obesity are associated with the maternal nutritional environment (2,3). In most mammals, fetal development is closely related to the maternal diet, and the genome of the preimplantation mammalian embryo undergoes extensive demethylation and remethylation processes that are extremely vulnerable to metabolic and environmental factors (4). DNA methylation plays an essential role in the establishment and maintenance of genomic imprinting, and while genomic imprinting affects mammalian genes that comprise only a small proportion of the genes in embryogenesis, these imprinted genes are critical regulators of fetal growth and development (5). Many studies suggest that DNA methylation can be affected by limiting the supply of methyl nutrients, resulting in a variety of diseases including neural tube defects, low birth weight and cancer (6–8).

Lipotropes are methyl group-containing essential nutrients (methionine, choline, folate and vitamin B\textsubscript{12}) that play key roles in one-carbon metabolism, a process that maintains the imprinting status of genes and provides methyl groups for all biological methylation pathways, including DNA methylation, which is correlated with controlling the expression of genes involved in cell growth, apoptosis and metabolism (9–11). Methionine and choline are major methyl donors, while folate and vitamin B\textsubscript{12} are critical cofactors for methyl metabolism (9,10). In mammals, one-carbon metabolism is dependent on dietary methyl donors and cofactors, and a maternal diet with methyl nutrients has been shown to influence DNA and histone methylation of the offspring’s genome (4).

As an epigenetic mechanism in cancer, DNA methylation affects the regulation of oncogenes and tumor suppressor genes (12). In tumor cells, global DNA hypomethylation promotes chromosomal instability and oncogene activation, while promoter hypermethylation silences tumor suppressor genes (13). DNA methylation is catalyzed by DNA methyltransferases, and histone acetylation levels are regulated by two classes of enzymes, the histone acetyltransferases and histone deacetylases (HDACs) (14,15). In humans, DNA methyltransferase 1 (DNMT1) binds to HDACs, which are recruited by methyl CpG-binding protein 2 (MeCP2) (16). MeCP2 or other methyl CpG-binding proteins can specifically bind to methylated CpG dinucleotides to repress transcription by forming the MeCP2–HDAC repressor complex (17). DNA methylation is linked to histone methylation and histone acetylation in gene silencing through the MeCP2–HDAC complex (16).

Maternal diet can influence the mammary development and differentiation that has been reported to be responsible for the protective effects against breast cancer (18). The growth of mammary glands mostly occurs during pregnancy and exhibits a steady exponential growth rate (19). Along with the allometric growth phase of the first pregnancy, increased methyl metabolism triggers a partial methylation of CpG sites in DNA and increases epigenetic reprogramming between the mother and her fetus (20). Hence, maternal methyl diet during pregnancy may create a stable epigenetic imprinting of genes in both dams and offspring that manifests as the mammary development and differentiation associated with decreased risk of breast cancer (21).

The primary aim of our study was to establish whether early life exposure to maternal high-dose lipotropes affects mammary carcinogenesis and epigenetic status in later life of the offspring. Such long-term effects of high-dose lipotropes on the development and progression of mammary tumors in offspring are not well known and there is considerable interest in the effects of in utero exposure to a high-methyl diet (single nutrient alone or in combination) on breast cancer risk. Our investigation consisted of two separate experiments: an in utero exposure only (Experiment 1) and an in utero exposure plus postnatal supplementation (Experiment 2). The purpose of both experiments was to test whether maternal dietary high-dose lipotropes affect the development and progression of mammary cancer in female rat offspring.

Materials and methods

Animal, breeding protocol and dietary treatment

All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of North Dakota State University. Virgin female Sprague–Dawley rats used for both experiments were purchased from Harlan (Madison, WI). All experimental diets were purchased from Harlan Teklad (Madison, WI). While the control diet was AIN-93G semipurified diet (22) prepared with basal levels of lipotropes (4.6 g L-methionine, 1.0 g choline, 2.0 mg folic acid and 25 μg vitamin B\textsubscript{12}/kg diet), the high-dose lipotrope diet was formulated to provide five times basal levels of lipotropes, except L-methionine was 1.8 times the basal level to avoid potential toxicity (23) (Supplementary Table S1, available at Carcinogenesis Online). The levels of lipotropes in the high-dose lipotrope diet were determined from our previous work and...
other studies (7,24). Weight and food intake were recorded weekly for all animals during pregnancy. The experimental feeding protocol is outlined in Figure 1.

Experiment 1 (in utero exposure). Female Sprague–Dawley rats (9 weeks of age) were acclimated to the experimental environment of ~25°C and a 12:12 h light–dark cycle for 1 week with ad libitum access to control diet. After acclimation, all female rats were randomly allocated to be fed either a control diet or a high-dose lipotrope diet (Supplementary Table S1, available at Carcinogenesis Online) and mated at ~10 weeks of age with proven-fertile male rats purchased from Harlan. Upon confirmation of pregnancy by the presence of a semen plug, 30 female rats (n = 15 per group) were singly housed and maintained on their respective diets until they delivered pups. Upon delivery of pups, the mothers were transferred to a control diet and the litters were randomly culled to a maximum of seven pups per litter. The pups received the control diet until they were weaned. The dams were killed using carbon dioxide asphyxiation and mammary tissues were collected for analysis. The breeding protocol described above was also used to collect mammary tissues from the offspring.

Experiment 2 (in utero exposure + postnatal supplementation). Female Sprague–Dawley rats (9 weeks of age) were acclimated to the experimental environment with ad libitum access to control diet. After acclimation, all female rats were randomly assigned to either control diet or high-dose lipotrope diet and mated with proven-fertile male rats purchased from Harlan. Upon confirmation of pregnancy by the presence of a semen plug, 20 female rats (n = 12 per group) were randomly chosen for mammary carcinogenesis (n = 12 per group) and pregnancy (n = 15 per group). The pups received the control diet until they were killed. The dams were killed using carbon dioxide asphyxiation at weaning, and mammary tissues were collected for analysis. The breeding protocol described above was also used to collect mammary tissues from the offspring.

Tumor induction and measurement

The single dose and intraperitoneal injection of N-nitroso-N-methylurea (NMU) during puberty (50 or 57 days of age) were used due to advantages such as short latent period, rapid initiation and high incidence of mammary tumors (25). NMU-induced primary rat tumors are mainly estrogen receptor positive and similar to low-grade human breast cancer (26).
acetyl-coenzyme A carboxylase alpha (Acaca, QT00190946), and gamma-glutamyl transferase 1 (Ggt1, QT00373072). The relative amounts of gene expression were standardized and calculated by the expression of housekeeping control genes, beta-actin (Actb, QT00193473) or glyceraldehyde-3-phosphate dehydrogenase (Gapdh, QT00199633) as an internal standard, using the 2^−ΔΔCt method.

**Global DNA methylation assay**

The mammary tumors and mammary tissues were collected and placed in RNAlater (Ambion) prior to freezing and then disrupted into small pieces. DNA was purified by the standard method using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma–Aldrich). Concentration was quantitated using a NanoDrop 2000c (Thermo Fisher Scientific) and global levels of cytosine methylation in DNA samples were measured using the Imprint Methylated DNA Quantification Kit (Sigma–Aldrich). Briefly, DNA samples (100 ng per well) were incubated with capture and detection antibodies, and then the absorbance was measured at 450 nm with a Spectra-Max Microplate Reader (Molecular Devices).

**Statistical analysis**

Kaplan–Meier survival curves and the log-rank test were used to analyze tumor incidence and survival rate. For the comparison of two groups with similar variance, a paired t-test was used. Differences between two groups (gene expression) were analyzed using a non-parametric t-test (Mann–Whitney test). Statistical data analyses were performed using Minitab Release 14.1 (Minitab, State College, PA). Differences were considered significant at P < 0.05.

**Results**

There was no significant difference in maternal weight gain between the two groups across the full pregnancy (Table I). Both groups displayed similar pregnancy rate, litter number, litter size and no postnatal death (Table I and Supplementary Table S2, available at Carcinogenesis Online).

**Experiment 1 (in utero exposure)**

*Mammary carcinogenesis in the offspring was significantly reduced by maternal high-dose lipotropes.* Palpable tumors were detected as early as 7 weeks after NMU injection in the offspring from dams fed a control diet (control offspring), and these rats had a mean tumor-free period of 8 weeks, whereas the offspring from dams fed a high-dose lipotrope diet (lipotrope offspring) had delayed tumor development and the mean tumor-free period was 11 weeks. As shown in Figure 2A, the control offspring reached 100% tumor incidence at 11 weeks after NMU administration, while the lipotrope offspring showed significantly increased latent period (+3 weeks, value was determined at 50% tumor incidence) and survival rate (+34%, P = 0.04, Figure 2D). The lipotrope offspring also displayed significantly decreased tumor incidence (−13%, P = 0.01, Figure 2A) and tumor multiplicity (−0.73 tumors/rat, P = 0.03, Figure 2B) at termination of the study. Furthermore, the lipotrope offspring also had significantly decreased total tumor volume of nearly 76.7% (P = 0.04, Figure 2C) at termination of the study. While tumor volume was greatly decreased in the lipotrope offspring, there was no significant difference in growth curves between the two groups during NMU carcinogenesis (Supplementary Figure S1A, available at Carcinogenesis Online).

**Gene transcription of MeCP2 and HDAC1 in mammary tumors was significantly reduced by maternal high-dose lipotropes.** Having observed reduced risk of mammary tumors in the offspring, we then investigated the messenger RNA (mRNA) expression of MeCP2, HDAC1, p53, Mdm2, Brca1 and Brms1 in mammary tumors by quantitative real-time PCR. As shown in Table II, the lipotrope offspring showed a statistically significant decrease in mRNA levels of MeCP2 and HDAC1 genes; however, p53 and Mdm2 showed no significant difference. The high-dose lipotrope diet also did not affect the expression of Brca1 and Brms1. Taken together, these results suggest that maternal high-dose lipotropes may reduce tumor cell growth by decreasing MeCP2 and HDAC1 gene expression.

**High-dose lipotropes did not affect the expression of mammary development and differentiation-associated genes in the mammary tissues of dams.** At the time of weaning (at 22 days of lactation), the dams were killed and mammary tissues excised. In order to determine if the maternal high-methyl diet influenced mammary development in the dams, we measured the mRNA expression of Csn2, Acaca and Ggt1 in mammary tissues by quantitative real-time PCR. As shown in Table III, although highly variable, there was no evidence that expression of any these genes in mammary tissues of dams was influenced by high-dose lipotropes. A similar pattern of gene expression was also observed in the lipotrope offspring.

**Global DNA methylation was not changed by high-dose lipotropes in mammary tumors and mammary tissues.** We measured global DNA methylation levels in mammary tumors and mammary tissues of dams and offspring using an enzyme-linked immunosorbent assay method. As shown in Supplementary Table S3 (available at Carcinogenesis Online), there was no difference in global DNA methylation in the mammary tissues from either dams or offspring. The high-dose lipotrope diet also did not change global DNA methylation levels in mammary tumors of offspring.

**Experiment 2 (in utero exposure + postnatal supplementation)**

*High-dose lipotropes significantly reduce mammary carcinogenesis in female rat offspring.* All female offspring were killed at 18 weeks after NMU injection for mammary tumor analysis. While the control offspring achieved a 100% tumor incidence at 12 weeks after NMU administration (Figure 3A), the lipotrope offspring showed significantly increased latent period (+5 weeks, value was determined at 50% tumor incidence) and survival rate (+33%, P = 0.03, Figure 3D). The lipotrope offspring also displayed significantly decreased tumor incidence (−25%, P = 0.01, Figure 3A) and tumor multiplicity (−1.25 tumors/rat, P = 0.01, Figure 3B) at termination of the study. Palpable tumors were detected as early as 7 weeks after NMU injection in the control offspring, and these rats had a mean tumor-free period of 8 weeks, whereas the offspring from dams fed a high-dose lipotrope diet (lipotrope offspring) had delayed tumor development and the mean tumor-free period was 11 weeks. As shown in Figure 2A, the control offspring reached 100% tumor incidence at 11 weeks after NMU administration, while the lipotrope offspring showed significantly increased latent period (+3 weeks, value was determined at 50% tumor incidence) and survival rate (+34%, P = 0.04, Figure 2D). The lipotrope offspring also displayed significantly decreased tumor incidence (−13%, P = 0.01, Figure 2A) and tumor multiplicity (−0.73 tumors/rat, P = 0.03, Figure 2B) at termination of the study. Furthermore, the lipotrope offspring also had significantly decreased total tumor volume of nearly 76.7% (P = 0.04, Figure 2C) at termination of the study. While tumor volume was greatly decreased in the lipotrope offspring, there was no significant difference in growth curves between the two groups during NMU carcinogenesis (Supplementary Figure S1A, available at Carcinogenesis Online).

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period of 8 weeks, whereas the lipotrope offspring had delayed tumor development and the mean tumor-free period was 13 weeks. The lipotrope offspring also showed an 84.9% (\( P < 0.04 \), Figure 3C) decrease in total tumor volume at the termination of the study. High-dose lipotropes significantly reduced \textit{Hdac1} gene transcription in tumor tissues. The mRNA levels of \textit{Mecp2}, \textit{Hdac1}, \textit{p53}, \textit{Mdm2}, \textit{Brca1} and \textit{Brms1} were analyzed by quantitative real-time PCR. A statistically significant decrease in the level of \textit{Hdac1} mRNA was observed in the lipotrope offspring; however, \textit{Mecp2}, \textit{p53}, \textit{Mdm2}, \textit{Brca1} and \textit{Brms1} showed no significant difference (Table II). These results suggest that high-dose lipotropes may be inhibiting mammary tumor development by decreasing \textit{Hdac1} gene expression.

\textbf{Discussion}

Breast cancer is a result of a series of oncogenic transformation processes including changes in the status of DNA methylation, known as an epigenetic alteration (12). Given that breast cancer can occur as both a genetic and epigenetic disease, some potential risk factors could be substantially modified by intrauterine epigenetic reprogramming (13). The purpose of this study was to determine whether or not a high maternal intake of lipotropes decreases mammary carcinogenesis in female rat offspring. Our results show that maternal high-dose lipotropes during pregnancy decrease both the expression of select DNA methylation-related genes and also the risk of developing carcinogen-induced mammary tumors in female offspring. Maternal high-dose lipotropes significantly reduced tumor incidence, tumor numbers and tumor volume, while also significantly increasing survival rate and tumor latency in the offspring (Figures 2 and 3).

In Experiment 1, maternal high-dose lipotropes caused a statistically significant decrease in the transcription of the genes \textit{Hdac1} and \textit{Mecp2} in mammary tumors of the offspring (Table II), but only \textit{Hdac1} was decreased significantly in Experiment 2 (Table II). Some studies have shown that, compared with normal cells, \textit{Mecp2} and \textit{Hdac1} are highly expressed in cancer cells (29,30). The HDAC1 protein represses gene transcription by recruiting MeCP2, and the expression of \textit{Hdac1} and \textit{Mecp2} genes is correlated with tumor initiation and development (17). However, maternal high-dose lipotropes did not change the mRNA expression of \textit{p53}, \textit{Mdm2}, \textit{Brca1} and \textit{Brms1} in either experiment (Table II). Investigating additional potential markers for mammary tumorigenesis and apoptosis will be needed in order to determine the molecular mechanism responsible for the suppression of carcinogenesis we observed.

Early first full-term pregnancy is associated with lower breast cancer risk and this protective effect is due to complete differentiation of...
the mammary gland characterized by a specific genomic signature imparted and induced during pregnancy (31,32). A pregnancy followed by breast-feeding in young women is associated with reduction in the lifetime risk of developing breast cancer, and additional pregnancies increase the protection against breast cancer (18). The protective effect of a full-term pregnancy is a well-established concept not only in humans but also in rodent models (20,33). Some rodent studies have shown that the neonatal exposure and postnatal supplementation of maternal high-dose lipotropes played a continuing role in further minimizing mammary tumor risk for the offspring. It may be that the methyl nutrient deficiency and supplementation on DNA methylation is gene and site specific and seems to depend on cell and organ types, along with the degree and duration of deficiency and supplementation (37). Investigating methylation patterns of individual CpG islands may prove to be useful in future studies.

Our results provide evidence that early life exposure to maternal high-dose lipotropes can reduce mammary tumor risk and affect epigenetic gene expression in later life of the offspring. Many case–control studies have also reported the protective effect of lipid tropic nutrients against dams’ or offspring’s breast cancer risk (38,39), and a study by Kovacheva et al. (24) demonstrated that maternal choline supplementation during pregnancy reduces breast cancer risk in offspring. One recent case–control study showed that there is a significant inverse association between folate/vitamin B₉ intake and breast cancer risk but no association with methionine/vitamin B₁₂ intake (39). In addition, a few in vitro studies have shown that lipotropic nutrients have inhibitory effects on human breast cancer cell growth (40,41).

Conversely, one animal study showed that maternal tumor risk of the offspring was not changed by maternal folate supplementation (four times) in NMU carcinogenesis (7), while a more recent study determined that maternal and post-weaning supplementation with folate (2.5 times) increases mammary tumor risk of the offspring in 7,12-dimethylbenz[a]anthracene carcinogenesis (42). The results of these single nutrient studies are somewhat contrary to the results of our study and there are some inconsistencies between single nutrient studies and combination studies. However, a normal diet has sufficient levels of all four lipotropic nutrients; thus, we believe that increasing all four in the diet may provide more relevant results given the complex molecular interactions involved in one-carbon metabolism.

The results from Experiment 2 showed a greater anticancer effect (tumor incidence, tumor multiplicity and tumor volume), which suggests that the neonatal exposure and postnatal supplementation of high-dose lipotropes played a continuing role in further minimizing mammary tumor risk for the offspring. It may be that the methyl metabolism that occurs in utero is continued to a lesser degree postnatally, but whatever mechanism is responsible for the decreased tumor growth appears to have used the methyl nutrients received after birth. Further studies investigating these various factors will be needed to clarify the mechanisms and circumstances responsible for suppressing mammary carcinogenesis.

This study demonstrated for the first time that maternal high-dose lipotropes are associated with reduced mammary tumor risk in the offspring. These findings may be a critical step in understanding the mechanisms between intrauterine dietary intervention and breast cancer risk and may be useful in developing potential clinical strategies to reduce breast cancer.

### Table II. Expression of CpG island, apoptosis and cancer-related genes in tumor tissues of the offspring

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<th>P values</th>
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<td>Mecp2</td>
<td>1.10 ± 0.41</td>
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<td>Hdac1</td>
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<td>p53F</td>
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<td>Mdm2</td>
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<td>Brmsl</td>
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### Table III. Expression of mammary development and differentiation-associated gene in mammary tissues (Experiment 1)

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<td>Csn2</td>
<td>1.45 ± 1.39</td>
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<td>Acaca</td>
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<td>Ggt1</td>
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<td>Csn2</td>
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<td>Ggt1</td>
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### Table IV. Expression of mammary development and differentiation-associated gene in mammary tissues (Experiment 2)

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<td>Mecp2</td>
<td>1.23 ± 0.42</td>
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<td>Hdac1</td>
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<td>Brmsl</td>
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Note: *Data are expressed as means ± SD (n = 5 per group). The relative amounts of gene expression were standardized and calculated by the expression of housekeeping genes, beta-actin and glyceraldehyde-3-phosphate dehydrogenase. **Mecp2 and Hdac1 were used as CpG region-related markers.  ***p53 and Mdm2 were used as apoptosis markers. ^Brca1 and Brmsl were used as mammary tumor markers. **Indicates a statistically significant difference between two groups of rats (P < 0.05).
Maternal lipotropes reduce offspring breast cancer risk

Fig. 3. Gestational exposure and postnatal supplementation of high-dose lipotropes (Experiment 2) decreased (A) tumor incidence, (B) tumor multiplicity (the number of tumors per rat) and (C) tumor volume (the average tumor volume of cumulative palpable mammary tumors) and increased (D) survival rate of the offspring. Female rats were randomly allocated and fed one of two experimental diets from gestation through lactation, and female pups received their respective diets based on the feeding regimen of the mother until they were killed. The offspring rats were given an intraperitoneal injection of N-nitroso-N-methylurea at 57 days of age. Data are expressed as means ± SEM (n = 12 per group). * * Indicates a statistically significant difference between two groups of rats (P < 0.05).

Supplementary material

Supplementary Tables S1–S3 and Figure S1A can be found at http://carcin.oxfordjournals.org/

Funding


Acknowledgements

We thank Jodie S. Haring, Robert Weigl, Kim C. Kraft, James D. Kirsch and Terry M. Skunberg for technical assistance.

Conflicts of Interest Statement: None declared.

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K.Cho et al.


Received November 3, 2011; revised March 12, 2012; accepted March 14, 2012