Parallel changes in metabolite and expression profiles in crooked-tail mutant and folate-reduced wild-type mice

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Anomalies in homocysteine (HCY) and folate metabolism are associated with common birth defects and adult diseases, several of which can be suppressed with dietary folate supplementation. Although supplementation reduces the occurrence and severity of neural tube defects (NTDs), many cases are resistant to these beneficial effects. The basis for variable response and biomarkers that predict responsiveness are unknown. Crooked-tail (Cd) mutant mice are an important model of folate-responsive NTDs. To identify features that are diagnostic for responsiveness versus resistance to dietary folate supplementation, we surveyed metabolite and expression levels in liver samples from folate-supplemented, folate-reduced and control diets in Cd mutant and wild-type adult females. Cd homozygotes had normal total homocysteine (tHcy) levels suggesting that folate suppresses NTDs through a mechanism that does not involve modulating serum tHcy levels. Instead, parallel changes in metabolite and expression profiles in folate-supplemented Cd/Cd homozygotes and folate-reduced +/+ and Cd/+ mice suggest that Crooked-tail homozygotes have a defect in the utilization of intracellular folate. Then, by combining these expression and metabolite profile results with published results for other models and their controls, two clusters were found, one of which included several folate-responsive NTD models and the other previously untested and presumably folate-resistant models. The predictive value of these profiles was verified by demonstrating that NTDs of Ski−/− mutant mice, whose profile suggested resistance to folate supplementation, were not suppressed with dietary folate supplementation. These results raise the possibility of using metabolite and expression profiles to distinguish folate-responsive and resistance adult females who are at risk for bearing fetuses with an NTD.

INTRODUCTION

Birth defects such as neural tube defects (NTDs) and adult diseases such as vascular disease, end-stage renal disease, cognitive dysfunction and certain cancers are often associated with anomalies in homocysteine (HCY) and folate metabolism (1,2). Disease pathogenesis is thought to result directly from the detrimental effects of elevated total homocysteine (tHcy) levels, folate deficiency or perhaps indirectly from redox reactions that are mediated through HCY, cysteine and glutathione metabolism (3). tHcy and folate levels are usually inversely related (1) and dietary supplementation with folate in many, but not all cases, lowers tHcy levels, restores normal folate levels and reduces the risk for NTDs (4), colon cancer (5)
and ischemic heart disease (6). Responsiveness to folate supplementation varies considerably for poorly understood reasons. With predicted responses based on genetic, molecular and metabolic features, dietary supplementation might be targeted to individuals who might benefit most and alternative treatments identified for folate-resistant cases.

NTD models in mice vary remarkably in their response to various dietary supplementations and nutrient treatments. Several models involving genes such as CART1 (7), CITED2 (8), Crooked-tail (9,10), FOLBP1 (11) and RFC1 (12) show reduced incidence and severity of NTDs after folate treatments, whereas other mutants such as axial-degeneration (13,14), bent-tail (15) and curly-tail (16) are resistant to the beneficial effects of folate supplementation. Responsiveness of some, but not all, of these mutants to dietary folate supplementation raises the possibility of identifying metabolite and expression biomarkers for differential response. These and other markers in functionally related metabolic and signaling pathways could then be tested for their relevance to NTDs in humans.

Crooked-tail (Cd) mutant mice are an important model of folate-responsive NTDs (9,10). Cd homozygous mutant mice show early lethality, exencephaly and small body weight in rare survivors; a crooked tail is found in Cd/+ heterozygotes (9). A diet supplemented with folic acid reduces early embryonic lethality and incidence of exencephaly in homozygous Cd/Cd mice (9). The Crooked-tail phenotype results from a mutation in the low density lipoprotein receptor-related protein 6 (Lrp6) gene, a co-receptor with Frizzled that is required for WNT signaling (10).

To identify diagnostic features that distinguish folate-responsiveness and -resistance in adult females, we undertook a systematic study of expression and metabolite levels in liver samples from Cd and wild-type female mice that were raised on a folate-supplemented, a folate-reduced diet or a control diet. We focused on liver samples from adult females partly because folate–HCY metabolism in liver has been extensively characterized in this organ and because risk for NTDs has in some cases been attributed to maternal metabolic anomalies (2). We found that profiles for folate-supplemented Cd/Cd homozygotes were indistinguishable and that folate levels were normal in Cd/Cd homozygotes, arguing that these mice, are functionally folate-reduced. Combining in a joint analysis, these results with those of a previous study for other NTD models (17), we discovered two main clusters of strains and mutants, one that was composed of mutants that respond to folate-supplementation and another whose responsiveness was unknown. We predicted that the second cluster included folate-resistant mice. Tests with one of these mutants, which involved an engineered deficiency of the SKI protein, verified these profile predictions.

RESULTS

We surveyed serum levels of tHcy and folate as key metabolic indicators of the HCY and folate pathways in wild-type (+/+), heterozygous (Cd+/+) and homozygous (Cd/Cd) females that were fed a control, folate-reduced or folate-supplemented diet. As expected (1,2), tHcy levels were elevated only in mice that were raised on the folate-reduced diet (Fig. 1A). Contrary to expectations, however, Cd mice had normal tHcy levels as well as normal levels of extracellular and intracellular folate compared with wild-types and heterozygotes, regardless of diet (Figs 1B and C). Therefore, the beneficial effects of folate treatments in Cd females were not mediated through a reduction in tHcy levels.

Two metabolites of HCY metabolism, total glutathione (tGsh) and total cysteine (tCys), were surveyed in mice of the various genotypes that were raised on the alternative diets. Glutathione (GSH) is a major cellular antioxidant and maternal glutathione may play an important role in protecting the embryo during early development by scavenging endogenous reactive oxygen species (18). tGsh levels were consistently elevated in Cd/Cd mice and tended to increase with decreasing amounts of dietary folate (Supplementary Material, Fig. S1), suggesting that the Cd mutation may adversely affect antioxidant functions in the liver. Cysteine plays numerous roles such as substrate for protein synthesis and probably as an extracellular antioxidant (19). However, no obvious patterns were observed for tCys levels among genotypes and diets (Supplementary Material, Fig. S2).

Hepatic expression profiles were used to identify genotypes and diets (as perturbations) that clustered together and to identify the patterns of metabolites and gene expression (as consequences of perturbations) that distinguished each cluster. Given the focus on folate and HCY metabolism, the miniarrays included most of the genes that are directly involved in folate and HCY metabolism as well as an equal number of unrelated control genes. Two main clusters of strains (A and B) and two main clusters of gene expression and metabolite profiles (1 and 2) were found (Fig. 2A). Cluster A included wild-type +/+ and Cd/+ mice on the control and folate-supplemented diets (4 and 10 mg FA/kg, respectively), whereas Cluster B included mutant Cd/Cd homozygotes on the control and folate-supplemented diets and wild-type +/+ and Cd/+ mice on the folate-reduced diet (0 mg FA/kg). Wild-type mice that were raised on the folate-reduced diet and Cd homozygous mutant mice that were fed the folate-supplemented shared several striking molecular features. For example, both showed significantly increased MTHFR and SHMT RNA levels and significantly reduced MTR RNA levels (Fig. 2A). Cluster 1 included genes in various metabolic pathways (HCY and folate metabolism, inflammatory response, lipid transport, phosphoinositide signaling and glucose transport and metabolism), whereas Cluster 2 included the remainder of the genes except those involved in phosphoinositide signaling and in glucose transport and metabolism. Jack-knifing, a resampling method that consists of iteratively deleting each unit of a data set (20), was used to test whether particular genes or pathways were critical for defining the clusters. Sequential elimination of each gene and separately each pathway did not affect the fundamental structure or composition of the various clusters, suggesting that clustering did not depend on results for particular genes or pathways (data not shown).

To test whether similarities among expression profiles might be diagnostic for responsiveness to folate supplementation, we compared expression profiles for the various Cd mice with those for Apob, Gli3, Pax3, Ptc and Ski mutant
Figure 1. Serum of total folate and HCY levels of wild-type, heterozygous and homozygous Cd female mice on control, folate-supplemented and folate-reduced diets. (A) Mean of total serum folate levels (pmol/ml): wild-type 0 mg, 7.0 ± 4.1, n = 15; Cd/+ 0 mg, 5.3 ± 2.4, n = 16; wild-type 4 mg, 72.2 ± 16.4, n = 8; Cd/+ 4 mg, 60.9 ± 12.5, n = 4; Cd/Cd 4 mg, 57.8 ± 14.4, n = 14; wild-type 10 mg, 54.5 ± 4.1, n = 5; Cd/+ 10 mg, 58.3 ± 21.5, n = 5; Cd/Cd 10 mg, 81.3 ± 18.4, n = 6. To assess differences in mean folate levels, the Newman–Keuls multiple comparison one-way ANOVA post-test was used. Values are expressed as mean ± SD. (B) Mean tHcy levels (μmol/l): wild-type 0 mg, 26.5 ± 16.5, n = 15; Cd/+ 0 mg, 13.2 ± 2.9, n = 14; wild-type 4 mg, 5.1 ± 0.9, n = 18; Cd/+ 4 mg, 4.5 ± 0.8, n = 13; Cd/Cd 4 mg, 4.7 ± 1.2, n = 13; wild-type 10 mg, 9.3 ± 4.0, n = 15; Cd/+ 10 mg, 8.1 ± 3.9, n = 15; Cd/Cd 10 mg, 8.1 ± 3.3, n = 6. To assess differences in mean HCY levels, the Newman–Keuls multiple comparison one-way ANOVA post-test was used. (C) Mean of liver tissue folate levels (pmol/mg protein): wild-type 0 mg, 1.0 ± 0.7, n = 16; Cd/+ 0 mg, 0.7 ± 0.4, n = 17; wild-type 4 mg, 8.0 ± 5.0, n = 10; Cd/+ 4 mg, 6.8 ± 2.5, n = 10; Cd/Cd 4 mg, 5.1 ± 2.0, n = 14; wild-type 10 mg, 5.5 ± 2.9, n = 10; Cd/+ 10 mg, 4.1 ± 2.4, n = 10; Cd/Cd 10 mg, 6.0 ± 4.2, n = 7. To assess differences in mean tissue folate levels, the Newman–Keuls multiple comparison one-way ANOVA post-test was used. Values are expressed as mean ± SD.
mice (Fig. 2B). Heterozygosity for these mutations adversely affects HCY levels and expression profiles for genes involved in HCY and folate metabolism (17). Interestingly, mice with phenotypic defects that are folate-responsive grouped together: one cluster contained \textit{Cd} (9,10), \textit{Apc} (21) and \textit{Pax3} (22,23) mutant mice, whereas the other cluster contained \textit{Apob}, \textit{Ptch} and \textit{Ski}, whose responsiveness to folate is unknown. These results predict that, despite having anomalies in HCY–folate metabolism (17), \textit{Apob}, \textit{Ptch} and \textit{Ski} mutant mice would be folate-resistant, or perhaps respond to folate treatments in a manner that is functionally distinct from the response of mutants in the first group.

Table 1. Effect of folic acid treatment on craniofacial and neural tube defects in \textit{Ski} homozygous mutant mice

<table>
<thead>
<tr>
<th>Strain and treatment</th>
<th>Total no. of offspring</th>
<th>No. \textit{Ski} \textit{−/−} (%)</th>
<th>No. of exencephaly (%)</th>
<th>No. of facial cleft (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6J</td>
<td>63</td>
<td>15 (24)</td>
<td>1 (6)</td>
<td>13 (87)</td>
</tr>
<tr>
<td>C57BL/6J + Folic acid</td>
<td>41</td>
<td>9 (22)</td>
<td>1 (11)</td>
<td>7 (78)</td>
</tr>
<tr>
<td>129</td>
<td>58</td>
<td>14 (24)</td>
<td>13 (92)</td>
<td>0</td>
</tr>
<tr>
<td>129 + Folic acid</td>
<td>39</td>
<td>8 (21)</td>
<td>8 (100)</td>
<td>0</td>
</tr>
</tbody>
</table>

Pregnant females were treated with folic acid as described in Materials and Methods. Folic acid treatment had no effect on the phenotypes of \textit{Ski-null} mutants (two-sided Fisher’s exact test: B6, $P = 0.8$, and 129, $P = 1.0$).
To test this hypothesis, we treated Ski mutant mice with folic acid during the first half of gestation. Ski mutants were tested in both the C57BL/6J (N9–N10 backcross generations) and 129S6 (N4 backcross generation) backgrounds to determine whether folic acid treatment reduced the incidence of either the clefting or exencephaly phenotypes observed in these strains (24). As predicted, folic acid had no effect on the incidence of either strain-dependent phenotype in Ski mutant homozygotes (Table 1).

**DISCUSSION**

Mutations in several genes in WNT signaling (Lrp6 and Apc) and the related sonic hedgehog (SHH) signal transduction pathway (Ski, Pch1, Gli3 and Pox3) cause NTDs and other birth defects as well as adult diseases associated with anomalies in folate and HCY pathways (25). Previous work showed that metabolite and expression levels for HCY and folate metabolism are adversely affected in mice that are heterozygous for mutations in the WNT and SHH pathways (17). Moreover, the ability of dietary folate supplementation to suppress phenotypes in many of these mutant mice and similarly folate deficiency to adversely affect SHH signal transduction suggests a reciprocal interdependency between these metabolic and signaling pathways. These interdependencies link purine and pyrimidine synthesis, amino acid metabolism, DNA, protein and lipid methylation in HCY and folate metabolism with cell adhesion, cell death, growth factors, differentiation, proliferation, positional information and transcriptional regulation in WNT and SHH signaling (1,2,25).

The striking similarities among the metabolite and gene expression profiles in folate-supplemented Cd/Cd mice and in folate-reduced +/+ and Cd/+ mice suggest that Cd homozygotes have a defect in folate utilization despite high folate levels in the diet, serum and liver. Cd/Cd cells either do not have adequate access to intracellular folate or are not able to utilize the available folate effectively. Perhaps, by increasing folate levels with dietary supplementation, a threshold is reached that restores normal neural tube development. Alternatively, perhaps folate utilization differs between fetal metabolism, when correction occurs, and adults, when diagnostic measurements are usually made.

NTDs are a classic example of multifactorial traits involving interactions between both genetic and environmental factors. Unraveling these complexities in humans is challenging. Because both genetic and environmental factors are readily controlled mouse models enable discovery of genes, molecules, pathways and dietary supplements that can be prioritized for testing in humans. The present study and published work (17) illustrates these complexities. For example, the cluster to which the Gli3-Xt mutation depended belonged on the inbred background on which it occurred (Fig. 2B). Similarly, the cluster to which the C57BL/6J (B6) strain belonged depended on which if any mutant was congenic on that background, with B6, B6-Apc<sup>min</sup> and B6-Pax3<sup>Sp</sup> included in cluster 1, but B6-ApoB and B6-Gli3<sup>xt</sup> included in cluster 2 (Fig. 2B). Thus, both the identity of the mutated gene and the genetic background influence gene expression and metabolite profiles. Moreover, folate deficiency may be a necessary but not sufficient condition to cause NTDs because folate-reduced wild-type mice do not show NTDs. Thus, mutations in Lrp6 and other genes in SHH and WNT signaling may be sensitizing mutations, whose phenotypic manifestations depend in part on genetic background and in part on folate levels in the diet.

The metabolic and gene expression profiles presented here identified two clusters of strains and mutants that may be generally predictive of folate acid responsiveness of neurulation on NTD-prone backgrounds. Combined with previous observations (17), adverse phenotypes associated with mutant genes in WNT signaling (9,10,21) and SHH signaling (22,23) can be suppressed with folate treatment. These pathways provide a promising system for genetic, metabolite and expression screening for NTD risk assessments in humans. Precisely, how the unique features of these profiles affect WNT and sonic hedgehog related protein functions and the general applicability of these profiles to other NTD defects and adult diseases associated with anomalies in HCY and folate metabolism remain to be determined.

**MATERIALS AND METHODS**

**Crooked-tail mutants: study population, diets and metabolite measures**

Female mice were used because in humans maternal HCY as well as fetal metabolism are risk factors for fetal NTDs (14). Virgin females were used to control for possible effects of pregnancy on HCY levels. An additional reason for focusing on females is that among human anencephalies and certain mouse exencephalies, such as Cd, females are more frequently affected than males (25). Mice were raised from conception on control [4 mg folic acid (FA)/kg chow], folate-supplemented (10 mg FA/kg chow) or folate-reduced (0 mg FA/kg chow) defined diets (P.J. Noyes, NH). Homozygous Cd/Cd mice on the folate-reduced diet were not analyzed because of their severely reduced viability (9).

**Cd genotyping.** The polymorphism detection protocol was described previously (9,10).

**Blood and tissue samples.** After deep anesthetization of 6–8-week-old virgin female mice, blood samples were obtained via cardiac puncture and were collected in nonheparinized tubes. After centrifugation, serum samples were stored at −80°C. At autopsy, liver samples were placed immediately on dry ice.

**Metabolite measurements.** The HPLC method of Ubbink and Vermaak (26) was used to measure total serum HCY, glutathione and cysteine. Total folate serum and hepatic levels were measured with a microbiological enzymatic assay (27).

**Ski mutants: study population and folate treatment**

Pregnant females were treated daily with folic acid (5 mg/kg body weight, by i.p. injection) from E0.5 (day of plug) to E9.5. Fetuses were collected at E16.5–E18.5, examined for craniofacial phenotypes and genotyped by Southern blotting (28).
Expression arrays

Arrays of gene-specific PCR products were used to evaluate the impact of mutation and diet on the RNA abundance of genes involved in HCY–folate metabolism as well as a panel of genetically and biochemically independent genes as controls. The methods that were used for making the arrays, gene-specific reagents, hybridization conditions and data analysis were described previously (17). Supplementary Material, Table S1 lists the genes that were included on the array.

Data analysis

We used two methods to analyze the expression data: hierarchical clustering (29) and mean-shift algorithm clustering (29,30). For the hierarchical clustering, the Multiple Viewer Experiment (MeV) software package developed by The Institute of Genome Research was used. The hierarchical analysis was statistically supported by jack-knife resampling with 1000 iterations, with resampling conducted on both strains and genes separately.

The data were also analyzed using the mean-shift algorithm (30,31), an unsupervised and non-parametric data density estimation procedure. Clustering is a natural application for the mean-shift algorithm. Each data point was assigned to the closest mode (cluster) center, starting with an arbitrary assumed mode center and then estimating the gradient of the data density function. At every step, we discovered that the assumed mode center is not really the center of the distribution of the mean in the direction of the gradient.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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REFERENCES