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Finding the Genetic Mechanisms of Folate Deficiency and Neural Tube Defects – Leaving no Stone Unturned

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Abstract

Neural tube defects (NTDs) occur secondary to failed closure of the neural tube between the third and fourth weeks of gestation. The worldwide incidence ranges from 0.3 – 200 per 10,000 births with the United States of American NTD incidence at around 3–6.3 per 10,000 dependent on race and socioeconomic background. Human NTD incidence has fallen by 35–50% in North America due to mandatory folic acid fortification of enriched cereal grain products since 1998. The US Food and Drug Administration has approved the folic acid fortification of corn masa flour with the goal to further reduce the incidence of NTDs, especially among individuals who are Hispanic. However, the genetic mechanisms determining who will benefit most from folate enrichment of the diet remains unclear despite volumes of literature published on studies of association of genes with functions related to folate metabolism and risk of human NTDs. The advances in omics technologies provides hypothesis-free tools to interrogate every single gene within the genome of NTD affected individuals to discover pathogenic variants and methylation targets throughout the affected genome. By identifying genes with expression regulated by presence of folate through transcriptome profiling studies, the genetic mechanisms leading to human NTDs due to folate deficiency may begin to be more efficiently revealed.

Keywords

human neural tube defects; folate one-carbon metabolism; genetic association; genetic variants; methylation

INTRODUCTION

A neural tube defect (NTD) is a congenital malformation of the central nervous system (CNS) occurring secondary to failed closure of the neural tube between the third and fourth

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weeks of gestation. NTDs affect approximately 300,000 livebirths worldwide every year and the incidence in different areas ranges from 0.03 to 20 per 1,000 births [Zaganjor et al., 2016]. The most common types of human NTDs occurring in approximately equal frequencies at birth are: spina bifida (SB) with lack of closure in the spinal cord region and anencephaly with lack of closure in the cranial region [Botto et al., 1999; Melvin et al., 2000]. Individuals with anencephaly usually die within days of birth. Advances in medical care and surgical procedures have greatly improved the survival of the majority of individuals affected by SB. SB is a broad term that includes myelomeningocele (MM), meningocele, and lipomeningocele. The most common SB is MM (protrusion of the nervous tissue and its covering through a defect in the vertebrae) accounting for more than 90% of SB cases. In this review the terms NTD and SB will be used throughout; however, most of the human genetic studies reviewed are primarily focused on MM. For the epidemiological studies that are discussed, NTD refers to all reported human NTDs, including anencephaly and SB.

Current NTD incidence in the United States (US) is around 3–6.3 per 10,000 live births annually dependent on race, geographic locations, socioeconomic background, and birth defects surveillance information available from each state [Parker et al., 2010; Zaganjor et al., 2016]. The estimated hospital care costs for a child born with SB range between \$21,900 and \$1,350,700 in the first year of life [Radcliff et al., 2012]. Intriguingly, the incidence of human NTDs fell by 35–50% in North America due to mandatory folic acid (FA) fortification of enriched cereal grain products in 1998 providing some relief to the large public health burden [CDC, 2009; Williams et al., 2015; Grosse et al., 2016; Noami et al., 2016]. However, NTD incidence in Hispanic Americans remains the highest among all races in the US. This population is the least likely to benefit from wheat flour fortification due to a preference for a corn masa flour-based diet [Hamner et al., 2013]. On April 15, 2016, the USFDA approved the FA fortification of corn masa flour to help increase FA intake for Hispanic American women of childbearing age with the goal of reducing the incidence of NTDs [DHHS FDA 21 CFR Part 172].

Despite reports from various parts of the world demonstrating reproducible results of the benefits of FA in reducing NTD occurrence and recurrence in the past two decades, the underlying molecular mechanisms remain unknown. There continues to be an estimated 30% of NTD cases that do not respond to FA supplementation [Crider et al., 2011]. In the past 25 years after the first discovery that NTD risk can be reduced with increased maternal folate uptake, there were extensive animal and human studies undertaken to facilitate our understanding of the genetics of NTDs. However, these studies have only enabled scientists to examine a limited number of genetic and epigenetic factors with minimal success in demonstrating association or pin-pointing molecular mechanism(s) on how folate one-carbon metabolism genes contribute to NTD risks in humans. Undoubtedly, in the “Omics” era, many exciting new tools including next generation sequencing will allow us to conduct hypothesis-free studies making it possible to interrogate every human gene to search for risk factors influenced by folate deficiency in NTD cases. Identifying specific genetic and epigenetic factors leading to human NTDs can enable health care providers to design knowledge-based gene-nutrition supplementation plans that may minimize NTD pregnancy risk. This review will summarize the genetics studies of human NTDs to provide a snapshot

of our current understanding of the biological mechanisms of folate to NTD development, specifically SB.

ENVIRONMENTAL FACTORS AND EPIGENETICS OF HUMAN NTDs

Expression of genes can be influenced by methylation of DNA, small RNAs and proteins. A review showed support that a wide variety of environmental factors (e.g. chemical pollutants, diets, temperature changes, and external stresses) can contribute to long-lasting effects on establishment and maintenance of epigenetic modifications that could thereby influence gene expression and phenotype [Feil and Fraga 2012; Yue et al., 2015; Price et al., 2016a]. A study of the methylomes in lymphocytes of human SB subjects showed significant overall hypomethylation of *SOX18*, a gene not known to associate with NTDs [Rochtus et al., 2016]. This study also showed that addition of *sox18* mRNA by injection into zebrafish embryos led to abnormal neural tube closure [Rochtus et al., 2016]. In addition, abnormal microRNA profiles were observed in human anencephaly brain tissue [Zhang et al., 2014] suggesting activity of microRNAs can be modified through post-transcriptional methylation [Berulava et al., 2015]. Many non-genetic/environmental factors in addition to folate have been demonstrated to contribute to NTD risk in numerous epidemiologic studies. For the past three decades, studies have investigated contributing risk factors including socioeconomic status; parental education [Grewal et al., 2008; Canfield et al., 2009; Brough et al., 2009]; maternal and paternal ages [Vieira and Taucher, 2005]; parental occupations [Shaw et al., 2002; Fear et al., 2007; Yang et al., 2008; Brender et al., 2010; Herdt-Losavio et al., 2010]; maternal reproductive history including maternal country of birth and country of conception [Canfield et al., 2009]; hyperthermia during early pregnancy [Moretti et al., 2005; Feldkamp et al., 2010]; glucose intolerance/diabetes or obesity [Shaw et al., 2003]; maternal intake of caffeine [Schmidt et al., 2009]; maternal medications [Alwan et al., 2007; Crider et al., 2009; Matok et al., 2009]; and toxic heavy metal exposure during early pregnancy [Mazumdar et al., 2015]. The non-genetic factors discussed above play some direct and indirect roles influencing the maternal and fetal epigenomes and increasing the risk of failed neural tube closure although the underlying molecular mechanisms are mostly unknown. Epigenetic factors may also vary with the genetic variations present in affected individuals, thereby influencing NTD risk [Greene et al., 2011; Price et al., 2016a].

GENETIC ETIOLOGY OF HUMAN NTDs

Genetic risk factors are important in NTD formation. First, individuals of Irish or Mexican descent have an a priori risk for NTDs which is higher than that of individuals of other Caucasian or of Asian descent [Ray et al., 2004; Njamnshi et al., 2008; CDC 2009]. Second, siblings of an NTD patient have a 3% or higher risk of having NTD, a 30 times increase over the population NTD risk of 0.1%, and offspring of second degree relatives has an increased risk of 0.5% [Toriello and Higgins, 1983]. Third, more than 200 mouse models of NTD, naturally occurring and genetically engineered, have been identified [Harris and Juriloff, 2007; Harris, 2009; Harris and Juriloff, 2010]. Fourth, about 1/5 of individuals with craniorachischisis were found to have deleterious variants in genes regulating planar cell

polarity [Harris et al., 2010]. Finally, a number of human syndromes have NTDs as a phenotypic feature suggesting genetic etiologies.

To date, over 300 studies attempting to find association of selected genes with human NTDs have been published. These studies, almost exclusively of simplex patients and their unaffected parents, have examined over 150 polymorphic variants in candidate genes with known functions involving various aspects of biological activity [Boyles et al., 2005; Greene et al., 2009]. The majority of the human association studies, both case-control and family-based, consist of MM cases together with other types of NTDs. Conventional genomewide linkage studies have rarely been undertaken because large, multigenerational families including multiple members affected with NTDs are uncommon. Genomewide association study (GWAS) has not been possible because human NTDs represent a rare complex trait precluding accumulation of a sufficiently large sample size in the thousands to achieve the statistical power necessary to detect an elevation of risk between 1 and 2. In addition, the statistical power for GWAS is further reduced with the diverse confounding factors present among patient populations with NTDs. Many recent studies have implicated rare genetic variants as contributing major effects in common complex diseases [Cirulli et al., 2010]. Indeed, approaches used to scan through sequences of candidate genes in exomes of affected subjects have identified rare deleterious genetic variants potentially contributing to some human NTDs [De Marco et al., 2014; Connealy et al., 2014; Lemay et al., 2015; Wang et al., 2015a; Ruggiero et al., 2015]. The impact of the FA fortification mandate on the penetrance of variants in genes rescuable by FA should be carefully weighed when designing studies to identify these variants. Enrolling study subjects, cases and controls, born before FA fortification period is necessary because controls without NTDs may include individuals with NTDs rescued by FA from food intake.

An alternative candidate gene association study approach based on findings from mutant NTD mouse models has produced interesting results in some cases [Harris and Juriloff, 2007]. A large fraction of the human NTDs and candidate gene association studies have focused on genes with pathways intersecting with the folate one carbon metabolism (FOCM) pathway in an effort to shed light on the underlying biological mechanisms for folate deficiency-induced human NTDs. Additionally, other etiological avenues have been investigated to aid scientists seeking answers to explain folate-resistant NTD cases. Here, we attempt to review studies of genes involved in FOCM and the interconnected pathways that code for the transporter/carrier of the metabolites and cofactors and the enzymes involving these biological cycles. Because of space limitations, all candidate genes discussed here are presented by the standardized gene symbol as recommended by the Human Genome Organization (HUGO). Details about candidate genes can be reviewed through the Entrez Gene database [<http://www.ncbi.nlm.nih.gov/gene/>]. We will provide an overview of these studies along with other current updates and discuss future directions to delineate the genetic etiology of NTDs.

REVIEW METHODOLOGY

Peer-reviewed articles are extracted from the PubMed/Medline. A list of genes coding for transporters and carriers of essential biomolecules for FOCM network were compiled from

NCBI-Gene [<https://www.ncbi.nlm.nih.gov/gene/>] using the term of the specific biomolecule with “transporter” and/or “carrier” (Tables 1). The FOCM network genes have been identified and described in the scientific literature and metabolic pathway studies providing the overall information to compile a list of genes as shown in Tables 2–6 [Litwack G 2008; Fox and Stover, 2008; Damaraju et al., 2008]. For this review, we searched for articles indexed in Pubmed/Medline using the term “human neural tube defects” and the name of each gene from the compiled list. Together, 30 FOCM network genes, 47 FOCM biomolecules transporting genes, 4 glycine cleavage system genes and 15 transsulfuration genes were searched for association with human neural tube defects studies. We used “methyltransferase” and “human neural tube defects” terms to identify articles reporting human NTD association studies on any methyltransferase (MTase) genes. This review aims to report which FOCM network genes have or have not been studied for association with human NTDs. Original articles and reviews reporting genetic studies of human NTDs regardless of study findings were included. Studies without human subjects affected by NTDs were excluded, such as studies reporting non-genetic factors or survey of NTD risk factors in the general population without NTDs.

GENES RELATED TO METABOLIC PATHWAYS OF FOLATE AND HUMAN NTDs

Folic Acid/One Carbon Metabolism and NTDs

Studies discussed in the previous sections led many researchers to test genes coding for proteins/enzymes in the one-carbon folate metabolism pathways for association with NTD risk. Folate one-carbon metabolism (FOCM) cross-regulates a complex interlocking network of biological pathways vital to maintenance, growth, differentiation, and proliferation of cells [Beaudin and Stover, 2007, 2009]. These pathways include folate synthesis and recycling, methionine/homocysteine (Met/Hcy) metabolism, transsulfuration and oxidative stress (ROS/GSH), purine and pyrimidine synthesis, serine/glycine synthesis, biomolecule methylation, membrane lipid synthesis, and drug metabolism (Figure 1). Formation of the neural tube involves intricately synchronized cell-cycle activities of both the cells composing the neural plate and the cells in the neighboring tissues (e.g. somites). Changes in genes may upset the balance of the aforementioned biological activities and disrupt the neural tube closing process resulting in NTDs [Beaudin and Stover, 2009].

Transport/Retention of Necessary Biomolecules and human NTDs

The FOCM cycle and the interlocking network of cycles involve many biomolecules that depend on transporters and carriers for cell uptake and enzymes to modify biomolecules for cell retention to sustain intracellular supply (Table 1). Failure to maintain a balanced supply to meet the biological needs of biomolecules (e.g. folate, methionine, glycine, serine, B6, B12, betaine and choline) can upset the FOCM network and subsequently lead to folate deficiency. Getting these necessary biomolecules from the intestinal lumen to cellular compartments involves a number of proteins produced by different genes. For example, folate transporter/receptor proteins code from *GCPII/FOLHI*, *SLC46A1/PCFT*, *FOLR1*, *FOLR2*, *FOLR3*, *SLC19A1/RFC1*, and *SLC25A32/MFTC*, each with specific tissue-

expression profiles. Upon cell entry, folate is quickly metabolized in different metabolic pathways. Folylpolylglutamate synthase (FPGS) and folylpolyglutamate hydrolase (GGH) work together to retain tetrahydrofolate (THF) within cells by converting THF to the polyglutamate form for utilization in the FOCM and Met. Multidrug resistance proteins listed in Table 1 (e.g. ABCC1-4, ABCB1-4, ABCG2, SLC22A6-8) known for transporting antifolates such as methotrexate may play some role in folate retention in cells [Damaraju et al., 2008]. Only a promoter polymorphism of *ABCC2* was tested for association with spina bifida risk in humans [Jensen et al., 2004]. Interestingly, a recent study has demonstrated that LDL receptor 2 (*Lrp2*) is essential for mediating folate uptake and folate deficiency in the *Lrp2* knockout mouse embryo contributing to NTDs [Kur et al., 2014]. Functional variants in genes coding for proteins transporting and/or retaining folate in cells may contribute to risk of NTDs in humans.

Methionine is an essential amino acid for humans, and it has been suggested to be protective for NTD risk in one association study [Graham et al., 2010]. Methionine from diet can be transported efficiently via amino acid carriers (e.g. SLC43A2, SLC7A5) into cellular compartments and is vital for synthesis of all proteins and to maintain the Met cycle. Mouse embryos with *Slc7a5* knocked out died with failure of the anterior neural tube to close and a cruciform caudal neural tube [Poncet et al., 2014], but it is not known whether the human homolog *SLC7A5* contributes to risk of NTD in humans.

Glycine and serine are two important biomolecules for the FOCM and Met/Hcy cycles and are transported into cells via several amino acid transporters (e.g. SLC32A1, SLC6A9, and SLC6A5 for glycine; SLC1A4, SLC3A2, and SLC7A10 for serine). Pyridoxal-5-phosphate is an active coenzyme for cystathionine- β -synthase (CBS) and converts homocysteine (Hcy) and serine into cystathionine for cysteine synthesis. Pyridoxal-5-phosphate is converted from vitamin B6 (pyridoxine) by intracellular pyridoxal kinase (PDXK) and pyridoxamine 5'-phosphate oxidase (PNPO).

The active form of cobalamin (vitamin B12), CH₃-B12, is a required coenzyme for MTR to convert Hcy and mTHF to THF and Met. B12 from the diet will be transported into cells by cubulin (CUBN) and transcobalamins (e.g. TCN1, TCN2, and CD320). Alternatively, oxidized B12 is converted to CH₃-B12 by MTRR to re-activate MTR activity. Alternatively, Hcy can be converted into Met by BHMT with betaine as a co-enzyme, and betaine can be transported into cells by different transporters (e.g. SLC6A18, SLC38A2, and SLC6A12). In addition, betaine can be converted from choline by CHDH and ALDH7A1 in mitochondria. Dietary choline can be transported into cells by several transporters (e.g. SLC5A7, SLC44A1, and SLC44A2) to synthesize betaine.

Only a small number of biomolecule transporters/carriers for folate and vitamin B12 (e.g. CUBN, TCN2, FOLH1, FOLR1-3, and SLC19A1) have been examined for association with NTD risk in humans [Franke et al. 2009; O'Byrne et al., 2010; Godbole et al., 2011; Pangilinan et al. 2012,]. These studies demonstrated that the transporters/carriers play a role in human NTDs and is consistent with the findings in mice that when the canonical folate transporters (e.g. Folh/Folr1, Slc19a1) are knocked down, the phenotypes are rescuable with a FA containing diet [Piedrahita et al., 1999; Gelineau-van Waes et al., 2008]. It is not

known whether other proteins that play a role in uptake or retention of the biomolecules involved in the FOCM network also contribute to NTD risk. Mouse embryos with the mitochondrial folate transporter (*Slc25a32*, alias *Mftc*) knocked down developed NTDs [Kim 2016]. In a targeted sequencing study of 239 subjects affected by spina bifida [Marini et al. 2011], a few new nonsynonymous variants were identified in three genes (*FOLH1*, *FGPS*, and *GGH*) with protein products that play roles in transporting and retaining folate in cells. Intronic variants were also identified. It is not known whether the new variants identified were deleterious to the function or expression of these genes. Unfortunately, the Marini et al. [2011] study did not examine other transporters or carriers of the biomolecules in the FOCM and related network. In another study, Franke et al. [2009] did not find association between *FGPS*, *FTCD* or *GGH* with human SB. In a companion paper by Findley et al. (this issue), deleterious variants are found in some other folate transporter genes (*SLC19A1*, *FOLR2*, *FOLR3*) in the exome of a small number of MM cases that may contribute to risk of MM. However, no new coding or splice site variants were found in some of the folate transportation genes (e.g. *FOLH1*, *FOLR1*, *SLC46A1* and *SLC25A32*). All the transporters/carrier genes of necessary biomolecules should be examined for deleterious variants to determine whether they may contribute to human NTDs. Therefore, expanding the search for association of the FOCM biomolecules transporters and human NTDs risks may be warranted.

Folate One-Carbon Metabolism Genes and Human NTDs

All of the genes in the folate metabolism cycle in the cytoplasm, mitochondria, and nucleus are obvious candidates for risk association studies of folate-responsive NTDs [Beaudin and Stover, 2009]. *DHFR*, *MTHFR*, *MTHFD1*, *MTR*, *MTRR*, and *TYMS* are among the most studied genes, and their mitochondrial counterparts are also gaining attention (Table 2). The most extensively studied is the *MTHFR* gene with over 100 published articles including a wide spectrum of populations [Boyles et al., 2005; Greene et al., 2009]. There is a large number of studies focusing on the *MTHFR* gene because the MTHFR protein converts 5, 10-methylene-THF (CH₂THF) to 5-methyl-THF (CH₃THF), the major intracellular form of folate utilized by both the FOCM and Met/Hcy cycles. A recent study found that CH₃THF was the most abundant form of intracellular folate in mouse embryos explaining why the CH₃THF producing enzyme MTHFR continuously attracts large interest in human NTD association studies [Leung et al., 2013]. Interestingly, over 90% of the *MTHFR* gene studies tested only two specific nonsynonymous SNPs (nsSNPs i.e., c.677C>T/p.Ala222Val and/or c.1298A>C/ p.Glu429Ala). Approximately half of these studies concluded that the c.677T genotype of the patient or the patient's mother increased NTD risk. Consistent with this finding, a large case-control study found only c.677C>T was associated with SB risk among 13 SNPs across the examined *MTHFR* gene locus [Shaw et al., 2009]. The MTHFR protein with variant p.222Val (c.677T) is thermolabile resulting in reduced enzyme activity that is consistent with MTHFR deficiency as a disease mechanism [Tsang et al., 2015].

Our group has identified an MTHFR promoter SNP (rs3737965) that is associated with SB risk using family-based reconstruction combined transmission disequilibrium test (RC-TDT) [Martinez et al., 2009]. A recent GWAS with a large sample size (4,763 women in the USA) found rs3737965 associated with lower plasma folate levels, but not elevated plasma Hcy

levels [Hazra et al., 2009]. The result for rs3737965 was a more significant p -value than that observed for c.C677T. Hazra et al. [2009] suggested rs3737965 could have a potential biological function similar to c.C677T. In another smaller study with Chinese subjects, rs3737965 was suggested to be associated with serum Hcy levels [Liang et al., 2014]. These findings suggest that SB risk can be associated with altered activity of MTHFR at the protein level and the transcriptional level. The SNP rs3737965 is not in linkage disequilibrium with c.677C>T or c.1298A>C and has not been studied by other groups in relation to human NTDs. The finding by Martinez et al. [2009] highlights the necessity of examining most, if not all, unlinked SNPs within each candidate gene locus including the promoter region, to get a true picture of how the gene is potentially involved in SB susceptibility.

Studies of other folate metabolism genes (*DHFR*, *MTHFD1*, *MTHFD2*, *MTR*, *MTRR*, *SHMT*, and *TYMS*) also focused on testing a few nonsynonymous SNPs (nsSNPs) within each gene or small insertion/deletion [indel] variants involving exons that might affect gene transcription [Boyles et al., 2005; Greene et al., 2009]. According to published studies, the *MTR* and *SHMT* genes have not been found to be associated with NTD risk. Positive association was concluded for the *TYMS* gene by testing several different SNPs in two studies, one that used a case-control design and another that used family-based RC-TDT design [Martinez et al., 2009; Shaw et al., 2009]. In these two studies, positive association was shown for one of the three SNPs tested in the *DHFR* gene in the family-based study, but negative for the nine different SNPs tested in a case-control study. Similar discordant findings were observed for the *MTHFD1* and *MTRR* genes with positive association found in the case-control study but no association detected in the family-based study. More thorough study of the *TYMS*, *DHFR*, *MTHFD1*, and *MTRR* genes is warranted.

Other folate pathway genes tested include *ALDH1L1*, *FTCD*, *MTHFD2*, *MTHFD1L*, and *MTHFS* [Boyles et al., 2005; Greene et al., 2009; Wu et al., 2016]. No significant association has been reported for *ALDH1L1*, *FTCD*, and *MTHFS*. Significant association was concluded for the heterozygous genotype, but not the homozygous genotype, for two SNPs of *MTHFD2* [Shaw et al., 2009]. A total of 118 SNPs and one ATT repeat found in the *MTHFD1L* gene were examined for association in Irish NTD patients. The TDT result demonstrated risk increased with (ATT)₇ and decreased with (ATT)₈ [Parle-McDermott et al., 2009]. Seven other SNPs out of the 118 tested demonstrated associations by case-control logistic regression analysis. The MTHFD1L protein is the mitochondrial counterpart of MTHFD1 in the cytosol for interconverting THF and CH₃THF. The MTHFD1L protein also produces the intermediate 10-formyl THF (fTHF) for the purine synthesis pathway. The important cellular function of this protein in folate metabolism and utilization provides a logical explanation as to the reason variation within the gene may be the key to susceptibility of NTD formation.

Marini et al. [2011] sequenced 239 subjects affected by spina bifida and found 29 new nonsynonymous/nonsense/frameshifting variants (allele frequency <0.5%) present only once among all subjects in 13 different FOCM genes except for *TYMS*. Intronic variants were also identified. Functional significance of the new variants identified has not been fully characterized. The study did not examine *DHFR1L*, *MTHFD1L* or *MTHFD2L*. With folate

being highly effective in reducing occurrence and recurrence of human NTDs by up to 70% in many published epidemiologic studies, the number of new variants identified in the FOCM genes (~12% of all subject tested) observed appears to be far less than anticipated in order to explain the effect of folate to reduce occurrence and recurrence of NTDs in these studies. Lack of deleterious coding variants identified in FOCM network genes may be explained by the presence of deleterious non-coding variants that have not yet been identified or these genes are highly sensitive to epigenetic effects resulting from folate deficiency [Feil and Fraga 2012; Berulava et al., 2015].

Purine and Pyrimidine Synthesis and human NTDs

Intermediates of the FOCM (i.e. fTHF and CH₂THF) are the basic building blocks for purine and pyrimidine biosynthesis. The enzyme phosphoribosylglycinamide formyltransferase (GART) is important in making phosphoribosyl-N-formylglycineamide (fGAR) from fTHF and glycineamide ribonucleotide (GAR), later converting to 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) for inosine monophosphate (IMP) synthesis by 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (ATIC). IMP is the common precursor for purine nucleotides AMP and GMP synthesis. FOCM intermediate CH₂THF is used as methyl group donor to deoxyuridylate (dUMP) to synthesize dTMP by TYMS for maintaining a balance of nucleotides supplied in cells. Association of SNPs in *GART*, *ATIC*, and *TYMS* with human NTDs risk remains inconclusive [Wilding et al., 2004; Shaw et al., 2009; Etheredge et al., 2012; Pangilinan et al., 2012; Piao et al., 2016]. New and known nonsynonymous variants were identified in *GART* and *ATIC* in patients with NTDs and were suggested to be associated with NTD risk in Hispanic subjects [Marini et al. 2011]. In addition to *GART*, *ATIC*, and *TYMS*, there are many other genes coding for enzymes in purine and pyrimidine biosynthesis. Whether or not other genes coding for enzymes participating in nucleobase biosynthesis can influence NTD risk in humans is largely unknown.

Methionine Cycle genes and human NTDs

Methionine is indispensable in translation of all eukaryotic proteins since all are initiated with a methionine codon. In humans, methionine is an essential amino acid, and the methionine pathway in conjunction with the folate pathway converts CH₃THF and Hcy to methionine, S-adenosyl-methionine (SAM), and S-adenosyl-Hcy (SAH). Many genes of the methionine cycle have been targeted to evaluate association to NTD risk (i.e. *AHCY*, *BHMT*, *BHMT2*, *MAT1A*, *MAT2A*, *MAT2B*, *MTR*, *MTRR*, and a few SAM-methyltransferases). So far, no association of NTD risk with SNPs in the *AHCY*, *BHMT2*, *MAT1A*, *MAT2A*, and *MAT2B* genes has been confirmed (Table 3). Association of *MTRR* with NTD risk has been demonstrated with mixed results [Shaw et al., 2009; Ouyang et al., 2013]. The association of c.742G>A (p.Arg239Gln) of *BHMT* and NTD risk remains unclear and needs to be replicated [Boyles et al., 2005; Greene et al., 2009; Martinez et al., 2009; Shaw et al., 2009]. Studies with expanded patient numbers, dense SNPs, replication in different patient populations, and multiple statistical methods will help clarify results.

A targeted sequencing study on subjects affected by SB has identified only a few new nonsynonymous variants of unknown significance in *AHCY*, *AHCYL2*, *MAT1A*, *MTR*, and

MTRR [Marini et al., 2011]. No new nonsynonymous variants were found in the other methionine cycle genes (*AHCYL1*, *BHMT*, *BHMT2*, *CHDH*, and *MAT2A*). The two methyltransferase genes *DNMT* and *GNMT* were not examined. Although variants in the introns were also identified it is not known whether these variants are deleterious to function or affect expression of these genes. It appears new coding sequence variants involving the methionine cycle genes do not play a major role in contributing to human NTD risk. So far, no additional studies have replicated the findings by Marini et al. [2011].

Glycine Cleavage System Genes and human NTDs

The glycine cleavage system (GCS), part of the glycine and serine catabolism pathway, plays an important role in mitochondrial FOCM. GCS is composed of four enzymes: glycine dehydrogenase (GLDC), aminomethyltransferase (AMT), glycine cleavage system protein H (GCSH), and dihydrolipoamide dehydrogenase (DLD). These four enzymes attach to the inner membrane of the mitochondria in response to high concentrations of glycine [Kikuchi, 1973]. Bi-allelic pathogenic variants of genes in GCS lead to the development of non-ketotic hyperglycinemia (NKH, OMIM #605899). NKH is an autosomal recessive inborn error of metabolism which causes accumulation of glycine and results in encephalopathy and often death [Azize et al., 2014; Kure et al., 2006]. Biallelic pathogenic variants in *DLD* have not been described among patients with NKH, and instead causes dihydrolipoamide dehydrogenase deficiency (DLDD OMIM #246900) with lactic acidosis and neurologic deterioration. More recent studies have shown a link between GCS genes in mitochondrial FOCM and human NTDs (Table 4) [Narisawa et al., 2012]. Mouse embryos with *Amt* knocked out develop NTDs with undetectable activity of GCS demonstrating AMT function is essential for GCS activity and successful neural tube closure. A link between NKH and NTDs was similarly established after a mouse model with *Gldc* knocked out also develop NTDs [Pai et al. 2015].

In a more recent study to identify the genetic etiology of subjects affected by MM, our group identified six novel heterozygous variants in the *AMT* gene and three of them (p.Val7Leu, p.Pro251Arg, and p.Val380Met) are predicted to be deleterious to AMT function [Shah et al., 2016]. In addition, five extremely rare known heterozygous variants were found in the *AMT* gene and one in the *GLDC* gene providing additional support that genetic variations in the GCS genes may contribute to the risk of human NTDs. No novel variants in the exons of the other two GCS genes *DLD* and *GCSH* were identified. Consistent with previous findings, deleterious variants in GCS genes in offspring play a small role in increasing NTD risk. As discussed above, the role of glycine and serine transporters should not be ignored since both amino acids can be derived from diet and transported into cells by their corresponding transporters.

Methylation and human NTD association

S-Adenosyl methionine (SAM), an intermediate biomolecule in the Met/Hcy cycle, is a major methyl group donor in transmethylation of a large spectrum of biomolecules (nucleic acids, proteins, phospholipids, amino acids, neurotransmitters and many other toxic compounds). Sequencing data from the Human Genome Projects has led to discovery of hundreds of human genes that contain coding domains similar to the characterized MTases

and catalogued by the Human Genome Organization (HUGO) Gene Nomenclature Committee HGNC (Table 5, http://www.genenames.org/cgi-bin/family_search?search=methyltransferase). One study suggested there are 208 methyltransferase (MTase) proteins [Petrossian and Clarke, 2011]. Thus far, more than 150 MTase genes have been identified in human genomes that contain a structurally conserved SAM-binding domain classified as SAM-dependent MTases (SAM MTases, IPR029063) in the InterPro protein families database (<http://www.ebi.ac.uk/interpro/entry/IPR029063>) [Martin et al., 2002; Schubert et al., 2003; Wlodarski et al., 2011; Mitchell et al., 2014]. Variation in function of various MTases that play important roles in growth, differentiation, and proliferation of cells may also be risk factors for susceptibility to NTD formation under folate deficient conditions [Blom, 2009; Crider et al., 2011; Imbard et al., 2013]. Many genes involved in transmethylation of nucleic acids (e.g. *AMD1*, *ATIC*, *DNMT1*, *DNMT3A*, *GAMT*, *GART*, *MGMT*, *RNMT*, and *TRDMT1*), proteins/amino acids (e.g. *ICMT*, *PCMT1*, *PRMT1*, and *PRMT2*), coenzymes and drug metabolism (e.g. *COQ3*, *NAT1*, *NAT2*, and *NNMT*), and lipids (e.g. *CHKA*, *MUT*, *PCYT1A*, and *PEMT*) have been examined for their potential contribution to risk of human NTDs, but the results were mixed [Boyles et al., 2005; Greene et al., 2009; Franke et al., 2009]. In a study of methylated DNAs from second trimester human placental tissues of NTD-affected patients and controls in Canada, differentially methylated array sites were identified between patients with SB and control individuals in the kidney, suggesting a common etiology for abnormal neural tube and renal development (n = 3342 sites) [Price et al. 2016b]. A review of the nutritional, biological, and genetic risk factors for NTDs in human and animal models led to a conclusion that folate and defects in the enzymes in FOCM individually are not sufficient to cause NTDs [Imbard et al., 2013]. Other components such as metabolism of choline, betaine, and B12 (which is essential for optimal methylation) and transmethylation are emerging as potential risk factors [Imbard et al., 2013]. With next-generation sequencing Omics tools, an in-depth evaluation of all MTase genes for association with human NTDs will shed light on what role MTases play in human NTD risk.

Methyltransferases for Nucleic Acids and human NTDs—DNA MTases (*DNMTs*) methylate DNA at the 5'-position of cytosine as the predominant epigenetic modification process in mammals. Methylation error can play a causal role in a variety of diseases, including cancer and NTDs [Robertson 2001; Rochtus et al., 2015]. In a chicken neural tube closure study, *DNMT3A* played a role in switching off neural tube transcription factor genes facilitating neural crest gene expression [Hu et al. 2012]. It has been suggested that methylation of genomic DNA is associated with risk of SB in China [Shangguan et al., 2015]. SNPs in *DNMT3A* have been demonstrated to be associated with NTD risk in humans with nominal significance [Pangilinan et al., 2012]. Mice with null *Dnmt3b* developed rostral neural tube abnormalities but *DNMT3B* has not been associated with human NTDs [Okano et al., 1999]. No association with human SB was found with other methyltransferases (i.e. *AMD1*, *ATIC*, *DNMT1*, *GAMT*, *GART*, and *MGMT*) targeting DNA [Franke et al., 2009].

Most recently, epitranscriptome studies have revealed modifications of RNAs by methylation (e.g. N6-methyladenosine or m6A, 5-methylcytosine or m5C, and pseudo-

uridine) at different locations. Regulation and function of these dynamic mRNA marks can determine splicing, nuclear export, capping, re-coding, translation efficiency, and stability [Yue et al., 2015; Gilbert et al., 2016; Popis et al., 2016]. The dynamic changes of the mRNA marks can affect cell fates, development, and disease [Klungland and Dahl, 2014; Wang, 2016]. Approximately 90 genes in the human genome are predicted to methylate different RNA species and the majority of them have not been fully characterized. In one study, nominal risk was suggested for the tRNA Aspartic MTase 1 gene (*TRDMT1*) and NTDs [Franke et al., 2009]. With *TRDMT1* located 13 kbp upstream of the vitamin B12 transporter gene, *CUBN*, it is possible that the observed associated risk is contributed by *CUBN*. On the other hand, Franke et al. [2009] did not find the RNA guanine-7-methyltransferase gene *RNMT* associated with SB. However, there are many more MTases targeting different types of RNAs in the human genome and whether these RNA MTases contribute to risk of human NTDs has never been studied.

Methyltransferases for amino acids and proteins and human NTDs—MTases targeting proteins (protein MTases) are broadly classified by the amino acid targets. Methylation reactions targeting lysine and arginine are the two largest subgroups encoding by more than 90 genes [Boriack-Sjodin et al., 2016]. Initial interest for protein MTases was mainly in studying the epigenetic effects of methylated histones and other proteins related to chromatid remodeling and their role in human disease [Kouzarides, 2007; Huang et al., 2008; Arrowsmith et al., 2012]. Aberration of protein MTases have been found in diverse cancer types. Previous studies suggested SNPs of *PCMT1*, encoding a Bax-induced apoptosis regulator L-isoaspartate (D-aspartate) O-methyltransferase 1, associated with increased risk for SB, and that the risk was further augmented by high level of maternal plasma Hcy [Zhu et al., 2006; Zhao et al., 2012; Wang et al., 2013]. No association for *ICMT*, *MGMT*, *PRMT1* and *PRMT2* with SB risk was observed [Franke et al., 2009]. However, the role of all other MTases in risk of human NTDs has not been fully examined.

Methyltransferases for lipids and human NTDs—In the PEMT pathway, SAM donates a methyl group to phospholipid phosphatidyl-ethanolamine (PE) to make phosphatidyl-choline (PC). PC and PE are main components of cellular membrane structures forming cellular organelles and cell-cell barriers. Metabolites from choline and phosphatidylcholine synthesis and degradation may influence cell cycle regulation and cell fate [Ridgway, 2013]. In addition, oxidation of choline produces betaine, a methyl donor of Hcy to synthesize methionine. A study found increasing intake of methionine may have a protective effect against NTDs [Graham et al., 2010]. Increased maternal intake of dietary choline has been suggested to reduce risk of NTDs. Mouse embryos exposed to choline uptake inhibitors developed NTDs [Fisher et al., 2001; Shaw et al., 2004; Lavery et al., 2014]. Choline kinase A (CHKA) and lack of choline intake have been shown to be associated with risk for human SB [Enaw et al., 2006]. A study by Pangilinan et al. [2012] showed *PEMT* was associated with NTD in the Irish population, but no association was found in patients in the USA [Zhang et al., 2006]. It is not clear whether genes coding for enzymes in the PEMT pathway or choline and betaine metabolism contribute to risk of human NTDs.

Other methyltransferases and human NTDs—Some studies have suggested environmental toxic chemicals such as arsenic compounds can lead to NTDs in animal model but the genetic mechanism is largely unknown [Ferm and Carpenter, 1968; Chaineau et al., 1990; Wlodarczyk et al., 1996]. So far, the association of toxic chemicals such as pesticides, mercury, lead, cadmium and arsenic with risk of NTDs in humans remains inconclusive [Brender et al., 2006; Jin et al., 2013]. Interestingly, a recent study with a small sample size has demonstrated that *AS3MT* and *DNMT3A* (MTases for arsenate and DNA, respectively) were associated with NTD risk in women with high plasma arsenates [Mazumdar et al., 2015]. Another study for ubiquinone, guanidoacetate, and nicotinamide methyltransferases (i.e. COQ3, GAMT, and NNMT) failed to demonstrate association with human SB risk [Franke et al., 2009]. Overall, the role of MTase function in detoxifying drugs and toxic heavy metals and human NTD risk has not been fully investigated.

Transsulfuration/reactive oxygen species reduction genes and human NTDs

FOCM and methionine cycles meet with MTR/MTRR-cobalamin converting Hcy and CH₃THFF to THF and methionine. High levels of maternal Hcy had been associated with increased risk for NTD-affected offspring. Other than the FOCM/methionine cycles, Hcy can convert into cystathionine in the presence of serine and vitamin B6 by CBS to reduce Hcy levels. Cystathionine can be used to synthesize cysteine by CTH and further into glutathione (GSH) by GSH synthase. GSH plays an important role in cellular antioxidant defense against superoxides with action of glutathione peroxidases (GPX). There are 8 different GPX genes in humans coding for GPX isozymes. These GPX isozymes are expressed in different tissues and organs with different peroxide substrate specificity. Only two genes (*CBS* and *CTH*) in this pathway have been tested for association with NTD risk (Table 6). The c.844ins68bp and SNP c.833T>C (rs5743905) of the *CBS* gene that potentially code for a truncated form of CBS have been studied by many groups on different patient groups with numbers ranging from 40 to 200 [Greene et al., 2009; Martinez et al., 2009]. In addition, a case-control study with 250 patients with NTD and 359 unaffected controls examined nine different SNPs spanning the 23 kbp *CBS* gene locus. Two SNPs (rs2851391 and rs234713) in intron 4 of *CBS* conferred modest risk for human NTDs [Shaw et al., 2009]. Only one study examined four SNPs of the *CTH* gene of 180 Dutch patients and found one SNP conferring risk [Franke et al., 2009]. The *CBS* and *CTH* genes function in transsulfuration and are good candidates to test for association to NTD risk. Study of correlation of other genes coding for transsulfuration enzymes and human NTD risk is lacking.

SUMMARY

Thus far the immense effort spent studying the FOCM/Met cycle genes to identify the genetic etiology of NTDs has not yielded proportionate results. The majority of the tested genes showed negative association with NTDs [Greene et al. 2009]. Positive associations with human NTDs have been suggested for some genes (i.e. *ALDH1L1*, *CHKA*, *CUBN*, *SARDH*, and *TRDMT1*) in a single study of a specific ethnic population that lack follow up replication studies for verification. Others studies demonstrated some genes (i.e. *BHMT*, *CBS*, *DHFR*, *DNMT3A*, *FOLH1*, *FOLR1*, *FOLR2*, *FOLR3*, *MTHFD1*, *MTHFD1L*,

MTHFR, *MTR*, *MTRR*, *PCMT1*, and *PEMT*) were associated with human NTDs only in some ethnic populations and appear to be in conflict with the overall effect that increased folate uptake reduces NTD incidence throughout the world. Inconsistent results reported between the case-control and the family-based studies are possibly secondary to sample variability, variable phenotypes, and small sample sizes. These conflicting results may be resolved with additional replication studies adjusted for confounding factors. There is insufficient evidence suggesting that the most commonly tested genetic variations leading to defects in the enzymes in the FOCM/Met cycles are the major molecular mechanisms contributing to rescue of the majority of human NTDs cases by folate supplementation. In fact, studies have shown that FA can alter expression of genes mediating cell growth, proliferation, and apoptosis, and these genes have not been shown to respond to FA availability [Price et al., 2016b]. The practice of FA fortification in food crops in the US has led to more than 35% reduction in NTD prevalence and effectively rescued a significant portion of individuals from having NTDs who carry deleterious FOCM network genes variants. As a result, the penetrance of deleterious variants in the FOCM network gene among subjects with NTDs after 1998 is expected to shift. Therefore, cautions must be taken to only select study subjects and controls born before 1998 in order to examine the role of FOCM network genes as risk factors for human NTDs.

Results of genetic association studies using common SNPs have been mixed. There is increasing evidence supporting a role for rare/novel/*de novo* variant in NTDs [Au et al., 2010]. Recent studies have suggested that while contribution of these variants in the PCP pathway genes contributed to more than 20% of severe human NTDs that involve the head (e.g. craniorachischisis), variants in these genes provided minimal contribution to the occurrence of spina bifida [Juriloff and Harris, 2012]. We have observed similar association for rare variants in several FOCM genes in a select group of MM subjects, and gene-disrupting *de novo* copy number variations (CNVs) might contribute to less than 8% of the etiology in MM subjects [Bassuk et al., 2013], both arguing for the need to examine a paradigm where rare *de novo* variants are potentially a major disease-causing mechanism of MM. In fact, several recent studies have reported identification of novel and *de novo* variants from subjects with human NTDs [De Marco et al., 2014; Lemay et al., 2015]. One must be cautious not to equate positive association studies with cause-and-effect relationship and variants with demonstrated association should be examined for functional significance if possible.

Leaving no stone unturned, whole exome sequencing (WES) provides an unbiased and hypothesis-free approach to examine the role of genetic variation in all of the genes connected to the FOCM/Met cycles. This approach has not been examined in previous association study designs because our knowledge of NTD is limited. Our group has an ongoing WES project including 500 patients affected with MM that is beginning to reveal MM-specific variants present in genes coding for receptor/transporter/carrier function and all enzymes related to FOCM pathway and the pathways related to the intermediate substrates/metabolites. With knowledge of the specific genetic variations of the MM exome and better understanding of how folate affects methylation and transcription of genes in embryonic cells, it is possible to formulate molecular mechanisms to explain the correlation of folate deficiency and risk of human NTDs. By expanding NTD risk association studies

beyond the FOCM/Met cycle genes, we will be able to assess the full extent of the influence that folate deficiency has on various genetic mechanisms that affect neural tube closure. Only with a knowledge-based evaluation of the involved specific genetic and epigenetic factors, will medical care providers be able to identify women of childbearing age who are mostly likely to benefit from folate supplementation to minimize risk of NTD-affected pregnancies. Concurrently, researchers can begin exploring other non-genetic factors as alternatives for the woman least likely to be responsive to folate supplementation to reduce risk of NTD-affected pregnancies.

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Figure 1. Folate one-carbon metabolism and interacting pathways components

Folate one-carbon metabolism (FOCM) and methionine cycle (Met cycle) is a major metabolic pathway involving many biomolecules involved in multiple metabolic pathways vital to cell survival, cell growth and proliferation, and cell differentiation. The participating biomolecules include different forms of folates, B-vitamins, betaine, choline, serine, glycine, threonine, and methionine for FOCM/Met metabolism, and glucose to generate energy for the metabolic processes. Biomolecules breaking down from food by maternal digestive enzymes will be absorbed by maternal enterocytes. Absorbed biomolecules will be transported into maternal circulation to supply maternal cells and organs and also to the developing embryo via the placenta during pregnancy. FOCM biomolecules transported into cells will be utilized by the FOCM/Met cycle enzymes producing important substrates for the downstream network of metabolic pathways including purine and pyrimidine synthesis, glycine cleavage, NADPH/NADP synthesis, peroxides transsulfuration, protein synthesis, phospholipid synthesis, and transmethylation of DNA, RNA and protein. Ser – serine, Gly – glycine, Thr – threonine, Met – methionine, Hcy – homocysteine, SAM – S-adenosyl-methionine, THF – tetrahydrofolate, fTHF – formyl-THF, CH₂THF – methylene-THF, CH₃THF – methyl-THF. B-vitamins: B2 – riboflavin, B6 – pyridoxine, B9 – folic acid or THF,

B12 – cobalamins, PE - phosphatidylethanolamine, PS - phosphatidylserine, PC - phosphatidylcholine.

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Table 1

FOCM network biomolecules transportation genes and NTD association studies

transporter/carrier genes*	biomolecules	NTD association study
<i>FOLH1, FOLR1, FOLR2, FOLR3, SLC19A1, SLC46A1, SLC25A32, ABCC2</i>	Folate, B9	Jansen et al., 2004; Relton et al., 2004a,b; Vieira et al., 2005; O'Leary et al., 2006; Pei et al., 2009; Shang et al., 2008; Doudney et al., 2009; Franke et al., 2009; Shaw et al., 2009; O'Byrne et al., 2010; Marini et al., 2011; Guo et al., 2013; Findley et al., 2016; Kim 2016; VanderMeer et al., 2016
<i>ABCC1, ABCC3, ABCB1-4, ABCG2-8, SLC04A1, LRP2</i>	Folate	none
<i>SLC7A5, SLC43A2</i>	Methionine	None
<i>SLC6A5, SLC6A9, SLC32A1</i>	Glycine	None
<i>SLC1A4, SLC3A2, SLC7A10</i>	Serine	None
<i>SLC52A1, SLC52A2, SLC52A3</i>	Riboflavin, B2	None
<i>PDXK, PDXP, PNPO</i>	Pyridoxine, B6	None
<i>CD320, CUBN, TCN1, TCN2</i>	Cobalamin, B12	Candito et al., 2008; Swanson et al., 2005; Franke et al, 2009; Godbole et al., 2011; Pangilinan et al., 2012
<i>SLC6A18, SLC38A2, SLC6A12</i>	Betaine	None
<i>SLC5A7, SLC44A1, SLC44A2</i>	Choline	Poncet et al., 2014

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Table 2

FOCM genes and NTD association studies

FOCM gene*	from	to	NTD association study
<i>ATIC</i>	fTHF, AICAR	THF, IMP	none
<i>ALDH1L1</i>	fTHF, NADP	THF, NADPH	Franke et al., 2009; Marini et al., 2011; Wu et al., 2016;
<i>ALDH1L2</i>	fTHF, NADP	THF, NADPH	Marini et al., 2011
<i>DHFR</i>	DHF	THF	Johnson et al., 2004; Parle-McDermott et al., 2007; Van der Linden et al., 2007; Doudney et al., 2009; Franke et al., 2009; Martinez et al., 2009; Shaw et al., 2009; Marini et al., 2011
<i>DHFR1</i>	DHF	THF	none
<i>FTCD</i>	THF, CH:NHTHF	fTHF, CH=THF	Franke et al., 2009; Marini et al., 2011
<i>GART</i>	5-phospho-D-ribosylamine, Gly, fTHF, ATP	5-amino-imidazole ribotide, THF Glu	Pangilinan et al., 2012
<i>MTHFS</i>	CH2THF, NADPH	fTHF	Franke et al., 2009; Marini et al., 2011
<i>MTHFR</i>	CH2-THF, NADPH	CH3-THF, NADP	>100 articles
<i>MTR</i>	CH3-THF, Hcy, CH3-B12	THF, Met, B12	Gos et al., 2004; Candito et al., 2008; Doudney et al., 2009; Franke et al., 2009; Shaw et al., 2009; Marini et al., 2011; Liu et al., 2014; Pangilinan et al., 2014; Wang et al., 2015b
<i>MTRR</i>	MTR-B12, SAM	MTR-CH3-B12, SAH	Zhu et al., 2003; Relton et al., 2004a,b; O'Leary et al., 2005; van der Linden et al., 2006; Candito et al., 2008; Doudney et al., 2009; Franke et al., 2009; Shaw et al., 2009; Marini et al., 2011;
<i>MTHFD1</i>	THF,ATP, formate	fTHF	De Marco P et al., 2006; McDermott et al., 2006; Van de Linden et al., 2006; Carroll et al., 2009; Doudney et al., 2009; Franke et al., 2009; Shaw et al., 2009; Marini et al., 2011; Pangilinan et al., 2012; Meng et al., 2015
<i>MTHFD1L</i>	fTHF	THF, formate	Parle-McDermott et al., 2009
<i>MTHFD2L</i>	CH2-THF, NADP	fTHF, NADPH	none
<i>MTHFD2</i>	CH2-THF, NADP	fTHF, NADPH	Franke et al., 2009; Shaw et al., 2009; Marini et al., 2011
<i>SARDH</i>	Sarcosine	gly	Franke et al., 2009; Marini et al., 2011; Piao & Guo et al., 2016;
<i>SHMT1</i>	CH2-THF, Gly	THF, Ser	Relton et al., 2004a,b; Franke et al., 2009; Marini et al., 2011; Etheredge et al., 2012
<i>SHMT2</i>	CH2-THF, Gly	THF, Ser	Marini et al., 2011
<i>TYMS</i>	CH2-THF, dUMP	DHF, dTMP	Volcik et al., 2003; Wilding et al., 2004; Franke et al., 2009; Martinez et al., 2009; Shaw et al., 2009; Marini et al., 2011; Etheredge et al., 2012

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Table 3

Methionine cycle genes and NTD association studies

Gene [*]	from	to	NTD association study
<i>AHCY</i>	SAH	Hcy, adenosine	Franke et al., 2009; Marini et al., 2011
<i>AHCYL1</i>	SAH	Hcy, adenosine	None
<i>AHCYL2</i>	SAH	Hcy, adenosine	Marini et al., 2011
<i>AMD1</i>	SAM	S-adenosyl-5'-3-methylpropylamine	Franke et al., 2009
<i>BHMT</i>	Hcy, betaine	Met, DMG	Zhu et al., 2005; Franke et al., 2009; Shaw et al., 2009; Liu et al., 2014
<i>BHMT2</i>	Hcy, betaine	Met, DMG	Franke et al., 2009; Shaw et al., 2009
<i>CHDH</i>	choline	Betaine	None
<i>MAT1A</i>	Met, ATP	SAM, ADP	Franke et al., 2009; Marini et al., 2011,
<i>MAT2A</i>	Met, ATP	SAM, ADP	Franke et al., 2009; Marini et al., 2011
<i>MTR</i>	CH3-THF, Hcy, CH3-B12	THF, Met, B12	Gos et al., 2004; Candito et al., 2008; Doudney et al., 2009; Franke et al., 2009; Shaw et al., 2009; Marini et al., 2011; Liu et al., 2014; Pangilinan et al., 2014; Wang et al., 2015
<i>MTRR</i>	MTR-B12, SAM	MTR-CH3-B12, SAH	Zhu et al., 2003; Relton et al., 2004a,b; O'Leary et al., 2005; van der Linden et al., 2006; Candito et al., 2008; Doudney et al., 2009; Franke et al., 2009; Shaw et al., 2009; Marini et al., 2011;
<i>DNMT</i>	SAM, DNA	SAH, CH3-DNA	See methyltransferase section.
<i>GNMT</i>	SAM, Gly	SAH, Sacrosine	See methyltransferase section

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Table 4

Glycine cleavage system genes and NTD association studies

gene	from	to	NTD association study
<i>AMT</i>	THF, CH ₂ NH ₂ -H-protein	CH ₂ =THF, H-protein(re)	Marini et al., 2011; Narisawa et al., 2012; Pai et al., 2015; Shah et al., 2016
<i>DLD</i>	NAD, H-protein(re)	H-protein(ox), NADH	Narisawa et al., 2012; Shah et al., 2016
<i>GCSH</i>	H-protein(ox)	H-protein(re)	Narisawa et al., 2012; Pai et al., 2015; Shah et al., 2016
<i>GLDC</i>	Gly, H-protein(ox)	CH ₂ NH ₂ -H-protein	Narisawa et al., 2012; Shah et al., 2016

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Table 5

Categories of Gene related to transmethylation in human genome

Potential target	Number of human genes	transmethylated molecule	Functional Significance
DNA	3	m5C of DNA	epigenetic marks, gene expression regulation
RNA	67	2'-O-methylation, m5C, m6A, m1G of RNAs	RNA processing, cleavage and stability, mRNA splicing, export and translation
Protein	101	lysine, arginine, N-methionine, C-leucine	chromatin remodeling, epigenetic marks, modify protein activity
others	20	catechols, histamine, Co-Q, NAD, serotonin, phospholipids, creatine, thiopurine, arsenites,	modify reactivity and availability of biomolecules, drugs and heavy metals

Table 6

Transsulfuration genes and NTD association studies

gene	from	to	NTD association study
<i>CAT</i>	H ₂ O ₂	H ₂ O	Davidson et al., 2008
<i>CBS</i>	Hcy, Ser, B6	Cystathionine	Relton et al., 2004a,b; Franke et al., 2009; Houcher et al., 2009 Martinez et al., 2009; Shaw et al., 2009; Tiley et al., 2012
<i>CTH</i>	Cystathionine	Cys	Franke et al., 2009
<i>GCLC</i>	Cys, Gln	Gln-Cys	None
<i>GPX1-8</i>	O ₂ ²⁻ , GSH, Selenium	H ₂ O ₂ , GSSG	None
<i>GSR</i>	GSSG	GSH	None
<i>GSTK1</i>	GSH, toxins	GS-Toxins, detox	None
<i>GSTM3</i>	GSH, toxins	GS-Toxins, detox	None

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