Published in final edited form as:
Dev Disabil Res Rev. 2010 ; 16(1): 6–15. doi:10.1002/ddrr.93.

Epidemiologic and genetic aspects of spina bifida and other neural tube defects

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Abstract

The worldwide incidence of neural tube defects (NTDs) ranges from 1.0 to 10.0 per 1,000 births with almost equal frequencies between two major categories: anencephaly and spina bifida (SB). Epidemiological studies have provided valuable insight for (a) researchers to identify nongenetic and genetic factors contributing to etiology, (b) public health officials to design and implement policies to prevent NTD pregnancies, and (c) individuals to take precautions to reduce the chance of having an NTD-affected pregnancy. Despite extensive research, our knowledge of the genetic etiology of human NTDs is limited. Although more than 200 small animal models with NTDs exist, most of these models do not replicate the human disease phenotype. Over a hundred candidate genes have been examined for risk association to human SB. The candidate genes studied include those important in folic acid metabolism, glucose metabolism, retinoid metabolism, and apoptosis. Many genes that regulate transcription in early embryogenesis and maintain planar cell polarity have also been tested as candidates. Additionally, genes identified through mouse models of NTDs have been explored as candidates. We do not know how many genes in the human genome may confer risk for NTDs in human. Less than 20% of the studied candidate genes have been determined to confer even a minor effect on risk association. Many studies have provided conflicting conclusions due to limitations in study design that potentially affect the power of statistical analysis. Future directions such as genomewide association studies (GWAS) and whole exome or even whole genome sequencing are discussed as possible avenues to identify genes that affect risk for human NTDs.

Keywords

spina bifida; meningomyleocele; neural tube defects; epidemiology; genetic association studies

Neural tube defect (NTD) is a general term for a congenital malformation of the central nervous system (CNS) occurring secondary to lack of closure of the neural tube with a worldwide incidence ranging from 1.0 to 10.0 per 1,000 births. The majority of cases can be categorized as either anencephaly (lack of closure in the region of the head) or spina bifida (lack of closure below the head; SB). The two categories of NTDs occur in approximately equal frequencies at birth [Botto et al., 1999; Melvin et al., 2000]. Individuals with anencephaly usually die within days of birth. Since 1960s, advances in medical care have

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led to the survival of the majority of individuals affected by SB. SB is also a broad term that encompasses several subgroups of defects including myelomeningocele (MM), meningocele, and lipomeningocele. Among these, MM (protrusion of the nervous tissue and its covering through a defect in the vertebrae) is by far the most common, accounting for greater than 90% of SB cases. In this review, the terms NTD and SB will be used throughout; however, most of the genetic studies reviewed are primarily focused on those individuals affected by MM. For the epidemiological studies that are discussed, NTD refers to all NTDs reported and this includes anencephaly and SB.

Epidemiological studies in the early 1990s discovered that maternal folate status is critical for proper neural tube closure during embryogenesis. These landmark studies showed that by increasing maternal folate levels, the occurrence and recurrence of NTDs could be significantly reduced [Czeizel and Dudas, 1992; MRC, 1991]. As a follow up to these reports, in January 1998, the United States Food and Drug Administration mandated fortification with folic acid of all cereal and grain products. By 2005, the prevalence of SB in the United States was two in 10,000 live births. Birth prevalence of SB in the United States decreased ~23% between year 1995–1996 and 2003–2004 showing the dramatic positive effect of food fortification with folic acid [Boulet et al., 2009]. Thus, fortification of foods with folic acid resulted in a significant reduction of the occurrence of SB, but it was not sufficient to completely abolish it. This suggests that factors other than maternal deficiency of folic acid are involved in the etiology of SB.

Although we know that maternal folate status is one important piece of the puzzle regarding why NTDs happen, we still do not understand the underlying biological mechanism. In the ensuing years since the discovery that maternal folate status was a key feature in NTD risk, extensive research has been undertaken to facilitate understanding of the process at the cellular level. Additionally, other etiological avenues have been pursued to aid researchers in finding answers to account for the NTD cases that are resistant to folate intervention. This review will summarize the epidemiological studies of NTDs as well as the genetics studies to provide a snapshot of where we are currently in our understanding of NTDs, specifically SB.

EPIDEMIOLOGY OF NTDs

Sociodemographic and epidemiological findings have been correlated with risk for an neural tube defect (NTD)-affected pregnancy. Many studies have investigated risk of NTD pregnancy for contributions of socioeconomic status (SES), parental education, maternal and paternal ages and occupations, maternal reproductive history including maternal country of birth and country of conception, hyperthermia during early pregnancy, hyperglycemia or diabetes or obesity, and maternal use of caffeine and medications during early pregnancy. We will summarize findings in the current literature on these issues.

Socioeconomic Factors

Increased risk for NTD offspring among households with low-SES, as measured by parental occupation and education and household income, has been reported in some studies [Brender and Suarez, 1990; Canfield et al., 1996a,b; Farley et al., 2002; Meyer and Siega-Riz, 2002] but not others [Strassburg and Greenland, 1983; Vrijheid et al., 2000]. A recent large scale study of births in California reported a simultaneous investigation of the role of individual and neighborhood SES characteristics to NTD risk in the postmandatory food fortification period. The report found women who did not graduate from high school and lived in low-SES neighborhoods exhibited a significantly higher risk for NTD pregnancy than women with high school or higher education who lived in the same neighborhoods [Grewal et al., 2008]. Another recent study reported a similar correlation of maternal

education status and NTDs among Hispanic Americans, especially for the less-acculturated Hispanic parents at highest risk of having offspring affected with NTDs [Canfield et al., 2009]. This finding may partially be explained by a recent report that mothers of higher social groups or with higher education were more likely to use folic acid in the preconceptional period and during the period of neural tube closure [Brough et al., 2009].

Parental Age and Birth Order

In a meta-analysis study of maternal age as risk factor for NTDs, the authors found increased risk associated with mothers of 40+ years and mothers younger than 19 years. The detected effect was stronger for SB than for anencephaly [Vieira and Taucher, 2005]. Perhaps somewhat related to maternal age, in a meta-analysis of birth order as a potential risk factor for NTDs, children with higher birth order were more likely to have SB [Vieira, 2003].

Parental Race

Recent estimates for the period of 2003–2005 in the United States found that the birth prevalence for NTDs per 1,000 births was 2.0 for non-Hispanic whites, 1.96 for Hispanics, and 1.74 for non-Hispanic blacks [Boulet et al. 2009]. Thus, there appears to be a slightly reduced risk for NTDs among non-Hispanic blacks in the United States. Among sub-Saharan blacks, the birth prevalence is 1.99 per 1000 births, which is higher than that observed for blacks in the United States [Njamnshi et al., 2008]. This increased prevalence among blacks in Africa is likely due to the lack of folic acid fortification. Before folic acid fortification, women from Latin countries and Northern China were at the highest risk for NTDs [Botto et al., 1999]. There have also been reports of increased incidence among the women of First Nations tribes in Canada [Ray et al., 2004] and among the indigenous women of western Australia [Bower et al., 2004] when compared with the nonindigenous women of those regions. Thus, there are clearly ethnic differences in rates of NTDs. However, the extent to which these differences are due to dietary factors (including consumption of folic acid fortified foods) versus genetic factors remains unknown.

Parental Occupation

A recent study found NTD pregnancy risk associated with self-reported multiple pesticide exposure [Brender et al., 2010]. The study result is consistent with a 1999 report of 207 cases in California [Shaw et al., 1999]. A 2002 study of 538 NTD cases occurring between 1989 and1991 in California suggests paternal occupations such as cooks, janitors and cleaners, farm workers, and gardeners are associated with increased risk to have a child affected with SB [Shaw et al., 2002]. Another study of 694 NTD cases in the United Kingdom between 1970 and 1987 suggested paternal occupational exposure to agrochemicals and animals was associated with NTDs. Interestingly, paternal occupational exposure to hydrocarbons and metal-working oil mists exhibited negative association with NTD risk [Fear et al., 2007]. A multicentered US case–control study of births between 1997 and 2000 also concluded paternal operator/laborer occupation was associated with SB [Yang et al., 2008]. A very recent report suggested that mothers in the janitorial/chemical semiconductor occupations have a higher risk for births of children with congenital anomalies including defects in the CNS [Herdt-Losavio et al., 2010]. More work will be needed to pinpoint the factors contributing to NTD pregnancy within the at-risk occupations.

Hyperthermia During Early Pregnancy

A meta-analysis of maternal hyperthermia in early pregnancy as a risk for NTDs found overall odds ratio of 1.92 for maternal hyperthermia [Moretti et al., 2005]. Single-ingredient-acetaminophen use during the first trimester for febrile illness does not increase

risk of major birth defects and may decrease risk of several malformations, including anencephaly and craniorachischisis, in a study of 11,610 cases and 4,500 controls delivered between 1997 and 2004 [Reldkamp et al., 2010]. This finding is consistent with observations reported in a large study of Spanish children that acetaminophen is not associated with an increased prevalence of congenital abnormalities [Rebordosa et al., 2008].

Maternal Caffeine and Medication Usage During Pregnancy

Increased risk of SB with increased total caffeine consumption during the year before pregnancy was reported in a study conducted 1997–2002. However, consumption of caffeinated tea showed a protective effect [Schmidt et al., 2009]. In a study in Israel, increased NTD risk was associated with exposure to one or more folic acid antagonists in the first trimester of pregnancy [Matok et al., 2009]. Preconceptional and first trimester usage of antibacterial medication was associated with increased risk of several birth defects included anencephaly but not SB [Crider et al., 2009]. Last, maternal use of selective serotonin-reuptake inhibitors was associated with an increased risk of anencephaly in a recent study [Alwan et al., 2007].

In summary, many clues concerning the underlying basis for risk of NTD offspring have been determined through epidemiological studies. Researchers worldwide have used these clues to dissect the genetic influences on NTD risk. The important roles of maternal folate and glucose status that were initially determined through epidemiological studies will be reviewed in the next section along with other genetic studies relating to NTD risk.

GENETIC ETIOLOGY OF SB AND NTDs

Several observations support genetic risk factors as important in NTD formation. First, a priori risk of NTDs for some ethnic/racial groups (e.g., Irish and Mexican) is higher than others (e.g., Caucasian and Asian). Second, NTDs recur within families, with first degree relatives of an NTD patient possessing a 3–5% risk of having offspring with an NTD and second-degree relatives a 1–2% risk. Third, more than 200 mouse models with NTDs have been described with some naturally occurring and others the result of genetic engineering in the laboratory [Harris and Juriloff, 2007; Harris, 2009]. Finally, a number of syndromes have NTDs as one phenotypic feature including single gene disorders as well as chromosomal disorders again, suggesting genetic etiologies. Unfortunately, conventional gene hunting strategies (i.e., positional cloning and genetic linkage mapping) are not applicable to identifying causative genes for NTDs, because, in the majority of cases, there is only one NTD-affected individual in a family. The patient family structure therefore limits study design strategies for discovering causative mutations in candidate genes associated with NTD risk. Several excellent detailed reviews of studies to identify genetic risk factors for NTDs can be found in Boyles et al. [2005] and Greene et al. [2009]. In this work, we are going to provide an overview of these studies along with other current updates and discuss future directions to delineate the genetic etiology of spina bifida (SB).

Over 130 studies attempting to find association of selected genes with NTDs were published between 1994 and 2010. These studies included ~132 candidate genes with known functions involving various aspects of biological activity (reviewed by Boyles et al. [2005] and Greene et al. [2009]). The samples in most studies consist almost exclusively of simplex (patients and their parents) families. The majority of the families included in the studies had one member with a MM, whereas a few of the studies included a small percentage of individuals affected with other types of NTDs. Large, multigenerational families including multiple members affected with NTDs are uncommon; therefore, conventional genomewide linkage studies using microsatellite markers have rarely been undertaken. Thus far, genomewide association study (GWAS) using human single-nucleotide polymorphism

(SNP) panels has not been possible, because no individual research group has a sufficient sample size to achieve the statistical power necessary to detect an elevation of risk between 1 and 2 for complex traits like NTDs. In addition, there are diverse confounding factors present between patient populations with NTDs that can further reduce the statistical power for GWAS.

The strategies used to select candidate genes for association studies of NTD risk are limited by prior knowledge of neural tube closure during early embryonic development. One popular idea has been to select candidate genes identified from mutant mouse models affected with NTDs [Harris and Juriloff, 2007]. Gene knockout (KO) technologies capable of disrupting specific genes in mouse greatly facilitated the discovery of genes causing NTDs in mouse models. From human studies, candidate genes were selected based on knowledge gained from epidemiological studies such as those that identified maternal folic acid deficiency and maternal derangement of glucose metabolism as associated with increased risk for NTD affected offspring. Folic acid (one-carbon) and glucose metabolism are two well-studied biochemical pathways; thus, each provides lists of candidate genes to be tested for association with NTD susceptibility. In addition, increased understanding of cell-cycle signaling genes regulating neural tube development has also provided potential candidate genes for NTDs. Because of space limitation, all candidate genes discussed here will be presented by the standardized gene symbol as recommended by the Human Genome Organization. Details about candidate genes can be reviewed through the Entrez Gene database (http://www.ncbi.nlm.nih.gov/gene/).

CANDIDATE GENES FROM METABOLIC PATHWAYS OF FOLATE AND GLUCOSE

Folic Acid/One Carbon Metabolism

Epidemiological studies discussed in 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 metabolism cross-regulates a complex network of basic biological pathways vital to growth, differentiation, and proliferation of cells [Beaudin and Stover, 2007, 2009]. These processes include folate recycling, methionine metabolism, transsulfuration, synthesis of purines and pyrimidines, synthesis of serine/glycine, biomolecule methylation, membrane lipid synthesis, and drug metabolism. Neural tube formation involves intricately synchronized cell-cycle activities of the cells composing the neural plate and neighboring tissues (e.g., somites). Abnormal activity of genes affecting the balance of the aforementioned biological activities can lead to failure of the neural tube to close appropriately resulting in NTDs [Beaudin and Stover, 2009].

Transport/retention of folate and vitamin B12—Failed uptake or failed intracellular retention of folate and vitamin B12 can lead to folate deficiency. Transportation of dietary folate to serum folate then to the cytoplasm of cells involves a number of proteins produced by folate transporter/receptor genes (i.e., *GCPII/FOLHI, PCFT, FOLR1, FOLR2, FOLR3*, and *SLC19A1/RFC1*) that each have defined tissue-expression profiles. Upon entering the cell, folate will be quickly metabolized in different metabolic pathways. Folylpolyglutamate synthase and folylpolygammaglutamyl hydrolase work together to retain and sequester both cytosolic and mitochondrial tetrahydrofolate (THF) by converting it to the polyglutamate form for utilization in the folate and methionine cycles. Association studies have been performed on a few selected SNPs located within these genes in different patient populations. It was concluded that no increased or decreased risk for NTDs was associated with the tested polymorphisms except for the G allele of c.80G>A (p.His27Arg) of *SLC19A1*. The G allele of c.80G>A of *SCL19A1* was associated with increased risk for

NTDs in Chinese, Italian, and Dutch populations, but not Irish, British, or American populations [De Marco et al., 2003; Pei et al., 2008].

Cobalamin and vitamin B12 are both required to activate MTR by MTRR in the folate/methionine cycle. Cubulin (*CUBN*) and transcobalamin (*TCNII*) are responsible for the cobalamin-B12 complex uptake by the cell membrane. No risk association for SB was found when testing SNPs of *TCNII* in multiple populations. A single study examined eight SNPs spanning across the 306 kbp locus of *CUBN* in 179 Dutch patients and 190 controls. One SNP (rs1907362) was found to associate with SB risk as well as red cell folate and vitamin B12 levels [Franke et al., 2009]. The observed positive result on *CUBN* supports the need to examine the candidate genes in depth among SB patients. Therefore, we should not exclude genes reported as showing no association, because most reported studies only examined a few SNPs within each gene that by chance may not be linked to disease causing mutations.

Folate Metabolism

Genes functioning in the folate metabolism cycle are obvious candidates for risk association studies for SB [Beaudin and Stover, 2009]. Among the most studied folate metabolism genes are *DHFR, MTHFR, MTHFD1, MTR, MTRR,* and *TYMS*. The *MTHFR* gene is the most extensively studied of all the folate metabolism candidate studies with 32 published including a wide spectrum of populations (reviewed by Boyles et al. [2005] and Greene et al. [2009]). The *MTHFR* gene is the main focus of so many studies, because MTHFR protein converts 5,10-methylene-THF to 5-methyl-THF (5-MTHF), the intracellular form of folate utilized by both the folate and methionine cycles. Interestingly, the majority (29/32) of the *MTHFR* gene studies tested only two specific nonsynonymous SNPs (nsSNPs) (i.e., c.677C>T/p.Ala222Val and/or 1298A>C/p.Glu429Ala) located within the gene. Sixteen of these studies concluded that the c.677T genotype of the patient or the patient's mother increased NTD risk. A large case–control study examined 13 SNPs across the *MTHFR* locus and found only c.677C>T associated with SB risk [Shaw et al., 2009]. MTHFR produced by a copy of the gene with p.222Val (c.677T) is thermolabile resulting in reduced enzyme activity consistent with functional loss of MTHFR as a disease mechanism. Martinez et al. [2009] recently reported another SNP (rs3737965) in the promoter of *MTHFR* associated with SB risk. The study used the reconstruction combined transmission disequilibrium test (RC-TDT) for analysis. In a recent genomewide SNP association study that focused on genes important in folate metabolism and that included a large sample size (4,763 US women), rs3737965 was found to associate with lower plasma folate levels but not elevated plasma homocysteine (Hcy) levels. The result for rs3737965 was a more significant *P*-value than that observed for c.C677T; however, the *P*-value was still below the genomewide significance level of 10^{-8} that had been selected for the study [Hazra et al., 2009]. A potential biological function of rs3737965 similar to c.C677T is implicated by the Hazra et al. [2009] study. 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. 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.

Reports of other folate metabolism genes (*DHFR, MTHFD1, MTHFD2, MTR, MTRR, SHMT,* and *TYMS*) also focused on testing a few nsSNPs within each gene or on small insertion/deletion (indel) variants involving exons that might affect gene transcription (reviewed by Boyles et al. [2005] and Greene et al. [2009]). According to the published studies, the *MTR* and *SHMT* genes have not been found to associate with NTD risk. Positive association was concluded for the *TYMS* gene by testing of several different SNPs in two studies, one that used a case–control design and another that used family-based RCTDT

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* (reviewed by Boyles et al. [2005] and Greene et al. [2009]). No significant association has been reported for *ALDH1L1*, *FTCD*, and *MTHFS*. Significant association was concluded for the heterozygous genotype but not 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 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 5-MTHF. The MTHFD1L protein also produces the intermediate (10-formyl THF) 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.

Methionine Cycle

Almost all eukaryotic gene translation initiates with methionine. Methionine is an essential amino acid in humans. The methionine pathway in conjunction with the folate pathway converts 5-MTHF and Hcy to methionine, *S*-adenosyl-methionine (SAM), and *S*-adenosyl-Hcy. Many studies have targeted genes of the methionine cycle to evaluate association to NTD risk (i.e., *AHCY*, *BHMT*, *BHMT2*, *MAT1A*, *MAT2A*, *MAT2B*, *MTR*, *MTRR*, and methyltransferases). So far, NTD risk has not been shown to associate with the SNPs tested in the *AHCY*, *BHMT2*, *MAT1A*, *MAT2A*, *MAT2B*, and *MTR* genes. Association of *MTRR* with NTD risk has been demonstrated with mixed results as previously discussed. The role of c.742G>A (p.Arg239Gln) of *BHMT* to NTD risk remains unclear with different studies providing opposing results [Boyles et al., 2006; Greene et al., 2009; Martinez et al., 2009; Shaw et al., 2009]. Further studies with expanded patient numbers, more SNPs, different patient populations, and multiple statistical methods will help clarifying the results.

Methylation

SAM is a major methyl group donor in transmethylation of biomolecules (nucleic acids, proteins, and lipids). Variation in function of various methyl-transferases that play important roles in growth, differentiation, and proliferation of cells may also be risk factors for susceptibility to NTD formation [Blom, 2009]. Studies have been carried out to investigate whether methyltransferases associate with NTD risk (reviewed by Boyles et al. [2005] and Greene et al. [2009]). Genes involved in transmethylation of nucleic acids (*AMD1*, *ATIC*, *DNMT1*, *DNMT3A*, *GAMT*, *GART*, *MGMT*, *RNMT*, and *TRDMT1*), proteins/ amino acids (*AMT*, *ICMT*, *PCMT1*, *PRMT1*, *PRMT2*, and *SHMT*), coenzymes and drug metabolism (*COQ3*, *NAT1*, *NAT2*, and *NNMT*), and lipids (*CHKA*, *MUT*, *PCYT1A*, *PEMT*, and *SARDH*) have all been studied for their potential role in causing NTDs. Association of a single SNP with risk for NTDs was reported for each of the following genes: the membrane lipid biosynthesis gene (*CHKA*), the drug metabolism gene (*NAT1*), and three of the protein methyltransferase genes (*ICMT*, *PCMT1*, and *PRMT1*). Nominal risk was suggested for the sarcosine synthesis gene (*SARDH*) and the tRMA methyl-transferase gene (*TRDMT1*) in reported studies. The fact that *TRDMT1* is ~13 kbp upstream of the vitamin B12 transporter

gene, *CUBN*, showed positive risk association to NTD may explain the nominal risk observed for *TRDMT1*.

Transsulfuration

Homocysteine and 5-MTHF are substrates for MTR in the folate and methionine cycles. High-maternal Hcy level had been associated with increased risk for many disorders including NTD affected offspring. Other than the folate/methionine cycles, Hcy can be converted to cystathionine by CBS in the presence of serine and vitamin B6. Cystathionine can be further converted to cysteine by CTH then to glutathione (GSH) by GSH synthase. In the presence of superoxides, GSH is converted to GSH disulfide by GPX. GSH has a major role in cellular antioxidant defense and detoxification. Only two genes (*CBS* and *CTH*) in this pathway have been tested for association with NTD risk (reviewed by Boyles et al. [2005] and Greene et al. [2009]). 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 patients groups with numbers ranging from 40 to 200. These studies have produced conflicting results. A current case–control study with 250 NTD cases and 359 unaffected controls examined nine different SNPs spanning the 23 kbp *CBS* gene locus and found two SNPs (rs2851391 and rs234713) in intron 4 conferring modest risk for NTDs [Shaw et al., 2009]. Another study examined four SNPs of the *CBS* gene on 608 SB patient families and found the c.833C>T (rs5742905) variant associated with SB risk using RCTDT [Martinez et al., 2009]. Only one study examined four SNPs on 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.

Glucose Homeostasis

Maternal diabetes, maternal obesity, and elevated daily glycemic index have been shown to be risk factors for NTDs, specifically SB, in various epidemiological studies [Waller et al., 2007; Correa et al., 2008; Stothard et al., 2009; Blomberg and Kallen, 2010]. One recent study found mothers with high-glycemic intake to have higher risk for NTD pregnancies even after adjusting for all confounding factors [Yazdy et al., 2010]. This study suggested that the hyperglycemic condition resulting from high-glycemic intake before and during pregnancy lie within the pathogenic pathway of NTDs. In the postfolic acid fortification era, it is increasingly important to identify causes of folate-resistant NTDs. Obesity and diabetes are growing problems in developed countries having taken on the potential of becoming epidemic. Obesity and diabetes are complex traits with multiple etiologies, both genetic and environmental. Understanding how these complex traits relate to NTD susceptibility remain an unknown but important area to be explored.

Glucose transport—Davidson et al. [2008] was the first to report association studies of genes functioning in glucose metabolism and risk of SB in the United States. Their study examined association of two glucose transporters, the *GLUT1* and *GLUT4* genes that were known to be expressed in placenta and the developing embryo, to SB risk in a large patient cohort. One nsSNP (rs2229682, p.Pro196Pro) in *GLUT1* associated with SB risk [Davidson et al., 2008]. The association was further supported in a follow-up study using 39 SNPs covering the entire 34 kbp span of the *GLUT1* locus that found nine SNPs with positive association. A few of these SNPs remained significant even after adjusting for multiple testing [Au et al., manuscript in preparation]. Hyperglycemia is known to affect expression and localization of glucose transporter proteins [Zhao and Keating, 2007; Pavlinkova et al., 2009]. There is one SNP in an intron of the *GLUT1*gene that is associated with risk for diabetic nephropathy. Additionally, mutations in *GLUT1* are known to cause the rare *GLUT1* deficiency syndrome (OMIM 606777) and a condition named paroxysmal exertion-induced dyskinesia (OMIM 612126). A mouse model with a transgenic *Glut1* antisense

clone knockdown expression of *Glut1* resulted in caudal regression and multiple defects [Helig et al., 2003]. These observations lead us to hypothesize that spatial–temporal impairment of *GLUT1* expression during neural tube closure is a mechanism for SB formation.

Other genes of glucose metabolism examined include *GAPDH*, *HK1*, *HK2*, *INS*, *INSR*, *LEP*, and *LEPR* with association demonstrated for one SNP in the *HK1* gene (p.Lys481Lys) as well as the *LEPR* gene (p.Arg109Lys) in a family-based study of 507 patient families [Davidson et al., 2008]. Glucose transporters, hexokinase, and the leptin receptor play different roles in diabetes and hyperglycemia. One group examined NTD risk associated with one microsatellite marker each for *LEP* and *LEPR* in a case–control study but found no association. The authors suggested that the small sample size might have contributed to the finding [Shaw et al., 2000]. More thorough examination for association of *HK1* and *LEPR* with SB risk is needed.

Energy metabolism—Glucose is the major source needed in cells to produce energy and maintain body heat through glycolysis. Two genes (*GAPDH* and *UCP2*) important in energy metabolism have been studied for SB risk association. No association was observed for SNPs tested located on *GAPDH* [Davidson et al., 2008]. Conflicting results were reported for *UCP2* in two independent case–control studies testing different polymorphisms with each study including less than 200 patients of different ethnic backgrounds [Volcik et al., 2003; Mitchell et al., 2009]. Replication studies are necessary to confirm or refute these findings.

CANDIDATE GENES BASED ON CELLULAR FUNCTION

Oxidative Stress/Apoptosis

Unregulated apoptosis of cells during neural tube closure due to oxidative stress may lead to NTDs. Genes that regulate oxidative stress include *CAT*, *NOS1*, *NOS2A*, *NOS3*, *SOD2*, *TXN2*, and *TP53*. Their function makes them all valid candidate genes for testing association with NTD risk. Studies to date have not found any association to NTD risk when testing SNPs on the *CAT*, *NOS1*, *NOS2A*, *NOS3*, and *SOD2* genes (reviewed by Boyles et al. [2005] and Greene et al. [2009]). Interestingly, a SNP in the promoter of *TXN2* was found to associate with risk in a case–control study that included 48 patients. This association needs to be verified with larger sample set. A large family-based study did not find risk associated with tested SNPs on *TP35*. However, another large case–control study of Irish patients with SB using 35 SNPs in the *TP53* locus demonstrated two SNPs of the patient genotypes associated with risk and two SNPs in the maternal genotype conferring risk [Pangilinan et al., 2008]. Replication of risk association of *TP53* in other samples should be performed to verify or refute these findings.

Retinoid Metabolism

Vitamin A and its derivatives (retinoids) have vital functions in vertebrate development and maintenance of various tissues. Retinoic acid (RA) is an active metabolite of vitamin A. Studies of the curly tail mouse, a naturally occurring mouse model of NTDs, indicated that RA might be an important candidate gene for NTD association studies in humans. The curly tail mouse is the best mouse model for the SB phenotype in humans, because no other NTD phenotypes are present. Among the 200 mouse models of NTDs, the majority exhibit defects in the head region making them less attractive for providing clues to the etiology of SB. In studies of the curly tail mouse, the hindgut endoderm, tail bud, and posterior neuropore region of ct/ct embryos showed reduced expression of RA receptors, suggesting potential involvement of the RA signaling mechanism in neural tube closure.

Several genes regulating metabolism of RA have been examined for NTD association in human including the *ALDH1A2, CRABP1, CRABP2, CYP26A1, CYP26B1*, and *RALDH2* genes, and no association was concluded in these studies (reviewed by Boyles et al. [2005] and Greene et al. [2009]). Replication study using more SNPs and in different populations are needed to verify or refute these provocative findings.

DNA Repair

Proper function of DNA repair mechanism in cells is critical to maintain normal cell activities and normal embryogenesis. Mice with DNA repair genes knocked out exhibit embryolethality with structural defects in offspring suggesting potential association of deleterious variants in DNA repair enzymes with risk of malformations. Some nsSNPs in several DNA repair genes (*APEX1, hOGG1, XPD, XRCC1*, and *XRCC3*) had been examined for association with SB risk in 123 patients with SB in a case–control study (reviewed by Boyles et al. [2005] and Greene et al. [2009]). A protective effect with the p.Asp148Glu variant of the *APEX1* gene and a mildly increased risk with the p.Lys751Gln variant of the *XPD* gene were reported [Olshan et al., 2005]. Again, replication of these findings in additional samples is necessary to make firm conclusions.

Gene-Expression Regulators/Transcription Factors

Gene-expression regulators/transcription factors genes (*HOX, PAX, MSX2, T brachyury, TFAP2a. ZICs, BRCA1, CITED2, TP53*, and *SLUG*) function to modulate expression of genes in many signaling pathways important for anterior–posterior body axis development. These genes have also been found to be essential in neural tube closure in animal models (reviewed by Copp and Greene [2010]). For example, the *Pax1* mutant (*undulated*) mouse exhibits various vertebral abnormalities. The homozygous *Pax3* (*splotch*) mutant mouse has SB and/or exencephaly and dies during gestation. Interestingly, risk association study testing SNPs in the *HOX* and *PAX* gene loci have not been reported. Volcik et al. [2002a,b] tested microsatellite markers near the *HOX* and *PAX* family genes for SB risk association using a family-based design. The microsatellite markers near the *HOX* family genes (*HOXA, HOXB, HOXC*, and *HOXD*) did not associate with SB risk. Association was found for the microsatellite markers near *PAX1*, *PAX7*, and *PAX8*, but not for *PAX2, PAX3, PAX5*, and *PAX6*. Resequencing of exons for *PAX1, PAX7*, and *PAX8* did not find significant disease-related variants in the study [Volcik et al., 2002a,b]. One study did report a risk associated SNP and haplotype after sequencing 4 of the 10 exons of *PAX3* in the DNA of 74 SB patients of varying ethnic background in the United States [Lu et al., 2007]. Other groups that have sequenced the two homeodomain-containing exons of *PAX3* in patients with a NTD did not find significant disease causing variants [Trembath et al., 1999].

Association studies examining the *BRCA1, MSX2, TFAP2a, ZIC1, ZIC2, ZIC3, CITED2*, and *SLUG* genes did not find risk associated SNPs (reviewed by Boyles et al. [2005] and Greene et al. [2009]). Studies on an intronic SNP of the *T. brachyury* gene demonstrated risk associated with Irish, Dutch, and UK patients, but not in German or US patients ([Shields et al., 2000]; reviewed by Greene et al. [2009]). Further replication will be needed to verify the reported associations.

CANDIDATE GENES BASED ON ANIMAL MODELS AND DEVELOPMENT GENES

Genes from Animal Models

Genes contributing to NTDs in animal models provide important information on selecting candidate genes for risk association study in human NTDs. SNPs in genetic loci including the *SHH, BMP4, NOG, CCL2, PRKACA, PRKACB, NAP1L2*, and *TERC* genes have been

examined for risk association with NTDs in humans, but no significant association has been reported (reviewed by Boyles et al. [2005] and Greene et al. [2009]). Several studies with small sample sizes examined SNPs and their haplotypes in the promoter region of the *PDGFRA* gene among SB patients (Dutch and Hispanic American). These studies suggested that the H1 haplotype conferred risk in heterozygotes [Joosten et al., 2001; Zhu et al., 2004]. Au et al. [2005] examined the same SNPs and haplotypes in a larger sample set including 407 patients with SB and their parents by TDT and did not find association.

Cell Recognition and Migration

Closing of the neural tube involves a cooperative migration and recognition of cells along the neural plate. Expression of the genes involved in these processes is critical to the successful neural tube closure process. Several genes (*CFL1*, *CSK*, *MACSK*, *MLP*, and *NCAM1*) important in cell recognition and the migration process have been examined for their role in contributing to risk of NTDs (reviewed by Boyles et al. [2005] and Greene et al. [2009]). No risk association was found with SNPs tested on the *CFL1*, *CSK*, *MACSK*, and *MLP* genes despite the fact that these genes are known to contribute to NTDs in mouse models. Association was demonstrated for four out of 11 SNPs examined in the *NCAM1* locus which is 317 kbp in size. All four SNPs demonstrating association were in linkage disequilibrium [Deak et al., 2005]. This finding suggests dysregulation of axon guidance and adhesion to be a possible disease mechanism for NTDs in human.

Planar Cell Polarity

The *loop-tail* mouse, which exhibits a severe NTD phenotype (equivalent to craniorachischisis in humans) with occasional SB, is caused by mutations in the *Vangl2* gene that functions in the noncanonical WNT signaling (planar cell polarity) pathway. An association study testing the noncanonical *WNT* pathway genes (*CELSR1-3*, *DSH*, *DVL2*, *DVL3*, *FZD3*, *FZD6*, *PRICKLE1*, *PRICKLE 2*, *SCRIB*, *VANGL2*, *WNT5A*, and *WNT11*) was reported in a very recent study with 338 SB cases and 338 unaffected controls of five ethnic backgrounds. No significant association was found after correcting for multiple testing [Wen et al., 2010]. The small sample size of each ethnic group was identified as a limitation to the study. A previous family-based study with 477 American Caucasian patients did not find association testing with SNPs from within the *DVL2* gene (reviewed by Boyles et al. [2005]). Sequencing of the *VANGL1* and *VANGL 2* exomes for patients with craniorachischisis and SB found no disease causing variants [Kibar et al., 2007]. A p.Val239Ile variant of *VANGL1* with reduced interaction to *DVL* has been reported in one patient with caudal regression syndrome. Mice with the *Vangl1* gene knocked out did not have NTDs. A combined loss of function of the *Vangl1* gene and another gene in the pathway is required to develop an NTD phenotype (reviewed by Greene et al. [2009]).

HUMAN NTD ASSOCIATION STUDIES AND DESIGN ISSUES

Limitations of Animal Models

The functional genes of humans and small laboratory animals are largely similar, providing the basis for using gene KOs in small animals to study human disease mechanisms and treatments. However, it is important that researchers keep in mind the differences observed between animal models and humans, so that necessary modifications can be made to better use animal models. This is particularly important for human diseases known to exhibit heterogeneity such as type 1 diabetes [Radbruch and Isaacs, 2009; von Herrath and Nepom, 2009]. More than 200 mouse models for NTDs have been studied, and it is questionable how well these models mimic the human problem.

Of the genes showing association with human SB, many have not been shown to result in SB or other NTDs in small animals when KO (e.g., *Cbs*, *Mthfd1*, *Mthfr*, and *Shmt*) and KO mouse models of some of these candidate genes result in embryonic lethal (e.g., *Mtr*, *Folr1*, and *Rfc1*) [Harris, 2009]. In Harris' review of NTD mouse models, none of the mouse mutants with folate-pathway genes KO had a phenotype like the majority of human NTDs. Association studies or direct sequencing of coding regions of human genes that show NTDs phenotypes in mouse models (i.e., *FOLR1*, *BMP4*, *DVL2*, *VANGL2*, and *NOG*) have failed to verify whether these genes contribute to risk of SB in humans (reviewed by Greene et al. [2009]). In addition, association studies of the genes that contribute to SB-only phenotype in mice: have either not yet been reported (i.e., *FKBP8* and *GRHL3*) or were reported and showed inconclusive results (i.e., *PAX3*). Certain neural tube closing sites observed in mouse have not been observed in humans [O'Rahilly and Muller, 2002]. The highly heterogeneous nature of human NTDs may explain the current slow progress when using mouse models to identify genes underlying human NTDs.

The significance of results observed in genetic association studies in human can be affected by the design of the study. Various study designs and potential affect on outcome of results are discussed below. Additionally, some ideas are presented to overcome the limitations of the designs.

Limitations of Human Studies

The majority of the published candidate gene association studies on NTDs are case–control studies with limited correction for population stratification. Studies with small sample sizes sometimes include subpopulations thus resulting in population stratification becoming a major issue in assessing true risk. When unmatched controls have been used, it is necessary to have two to fourfold controls to affected samples to correct for effect of mismatched factors. This is labor intensive and often not done.

Approximately 30% of the published human NTD association studies used family-based statistics. Family-based studies generally require larger sample sizes; however, the study design is robust in accounting for confounding effects such as population stratification. Furthermore, in family-based studies, there are other statistical methods (e.g., log-linear regression and likelihood ratio test) that help with differentiating whether the risk allele is in the patient's genotype or the mother's genotype [Weinberg et al., 1998]. It is particularly important in complex common birth defects to account for maternal effect as the defect occurs while in the in utero environment provided by the mother.

As discussed in several recent reviews on genetics of NTDs, many of the published association studies are statistically underpowered, providing one possible explanation for conflicting results reported among studies (reviewed by Greene et al. [2009], Harris [2009], and Copp and Greene [2010]). Of the 132 published human NTD association studies, ~9% have a sample size of more than 500 patients, 18% include more than 400, and 30% have more than 250. In all, roughly 25% of the 132 published NTD association studies concluded their findings from a patient cohort of less than 100. Adding to the problem of low power, factors that make the samples more heterogeneous (e.g., variations in SES and ethnicity) weaken the capacity of different studies to accurately assess NTD associations. Therefore, it may not be accurate to include or exclude risk contribution of the tested genes from these studies. In general, a total of 1,000 cases and 1,000 controls are needed to provide statistical power of 80% to assess a risk of 1.8–2 of a disease locus with a SNP allele frequency of 0.1. Double or quadruple the controls will be needed if unmatched controls are used to adjust for confounding factors. For complex disease like NTDs, it is anticipated that the risk of a disease associated allele to be between 1 and 2 and over 2000 samples will be needed to

detect small associations of the type characteristic of NTDs in humans with 80% statistical power.

For example, a patient cohort of 550 should be sufficient to detect risk of 2.0 of a disease allele (minor allele frequency of 0.1) with a statistical power over 95% using the family-based RC-TDT in multiplicative, additive, and dominant models. The statistical power will reduce dramatically when the disease locus minor allele frequency falls below 0.1 and many more SB patient families will be needed to maintain the statistical power.

Most early studies tested a few known SNPs deduced to have a high likelihood of affect on protein function (i.e., nsSNP or indel in untranslated regions). Only a few recent studies used tagged SNPs to cover the major genotype blocks of the tested candidate genes. Only 16 genes in the 132 reported studies had a density of one SNP for every 2 kb of sequence along the tested candidate genes (*CCL2/MCP-1*, *CFL1*, *CRABP1*, *CRABP2*, *CRABP2*, *CYP26A1*, *CYP26B1*, *DVL2*, *FOLR2*, *INS*, *MSX2*, *MTHFD1L*, *MTHFR*, *NNMT*, *TERC*, and *TP53*). Testing of only one or two SNPs per gene in the NTD association studies may also contribute to the lack of replicable findings. Testing only one SNP within a candidate gene and hoping it links to other SNPs within the locus is unpredictable and provides little information regarding whether the candidate gene associates with NTD susceptibility. The current SNP arrays used in GWAS include a million SNPs with density near one SNP per kbp. Furthermore, the majority GWAS SNPs are tagged SNPs that cover over 95% of linked markers.

Between 1995 and 2009, the exons of a total of 18 genes (*CYP26A*, *FOLR1*, *MLP*, *MSX2*, *MTHFD1*, *MTHFD1*, *NOG*, *PAX1*, *PAX3*, *PRKACA*, *SHH*, *SHMT*, *SLUG*, *RFAP2A*, *VANGL1*, *VANGL2*, *ZIC1*, *ZIC2*, and *ZIC3*) were examined and reported in 22 studies attempting to discover disease causing mutations for NTDs cases (reviewed by Boyles et al. [2005] and Greene et al. [2009]). Sequence variants were identified in some of the genes (i.e., *CYP26A*, *FOLR1*, *MSX2*, *MTHFD1*, *PAX1*, *PAX3*, *SHH*, *VANGL1*, and *ZIC2*), and these variants have been implicated as causes of NTDs in humans. However, only the p.Val239Ile variant in the *VANGL1* gene has been shown to result in loss of protein function. Studies of large samples are needed to determine whether variants are important in NTD susceptibility or simply benign private polymorphisms.

FUTRE DIRECTIONS FOR NTD ASSOCIATION STUDIES

In addition to the issues raised in the previous section, a review of publications reporting association studies of human NTDs shows that 42 of the 132 candidate genes tested were associated (significantly/nominally) with or protect from NTD risk. Furthermore, among the 42 candidate genes identified as significant in the published studies, 17 were found positive in underpowered studies (those less than 150 NTD patients). Therefore, there are currently 25 candidate genes (see Table 1) that merit follow up in replication studies performed using samples from different populations. It is essential for future replication studies to be designed with large sample sizes and matching controls to minimize the effect of confounding factors. Correction for the effects of factors associated with increased heterogeneity and variability in NTDs/SB (e.g., SES, ethnicity) and adjustments for Type I error rates associated with multiple-dependent statistical tests should also be included as a routine practice in evaluating the associations of genetic factors and risk for NTD/SB.

The “hypothesis-free” GWAS approach has been used to screen for genes associating with complex diseases such as type 2 diabetes. These studies have provided positive results and discovered unexpected disease gene candidates [Prokopenko et al., 2008]. However, successful GWAS requires sample sizes of 2,000 or more patients and controls to provide sufficient statistical power to detect a risk of one to two. Because NTDs are far less common

than diabetes, GWAS studies are impossible for any individual research group. For NTDs, a consortium of research groups pooling sample sets is necessary to make the GWAS approach possible. Using the GWAS approach to identify NTD associated genes has the advantage of examining the genes corresponding to all of the ~200 NTD mouse models as well as all other genetic loci in the human genome linked to the SNPs in the GWAS SNP-array. Candidate gene association approaches are not nearly as inclusive and comprehensive.

Multiple laboratories have been engaged in selective resequencing of exons of genes with demonstrated association by association studies with the goal to discover potential disease causing sequence variants for patients with SB. However, the current Sanger sequencing approach is relatively costly and is limited to detecting variants within and 50-bp flanking the exoncoding sequences. This methodology will miss the discovery of noncoding functional variants in the introns or promoter region that may alter expression or splicing of the gene. Using the high-throughput sequencing approach modified to capture and sequence target genetic loci for a representative subset of patients can greatly facilitate the discovery of coding or noncoding patient-associated variants. Private variants identified from patients with SB can be used in expanded follow-up association studies and for functional studies to verify their role in the pathogenic process. A new approach that captures the exons of all the genes in the human genome (whole exome) has been successfully used to identify causative genes for rare single-gene disorders. The potential to apply this technology to complex traits remains to be seen.

A limitation of the conventional candidate gene approach to identify genome sequence variants associated with NTD risk is its inability to identify long-range transcription regulatory elements for candidate genes. An increasing number of long-range transcriptional regulatory sequences have been identified such as the G allele of rs6983267 located 395 kbp upstream of the *c-Myc* gene that acts as transcription enhancer for *c-Myc* expression in colorectal cancer cells. This regulatory sequence was identified through the Chromosome Conformation Capture approach [Pomerantz et al., 2009]. Alternatively, association study designs using the Illumina Human 1M array can potentially identify positively associated SNPs located far away from genes and potentially linked to long-range transcription elements of candidate genes. SNPs identified by GWAS can then be further studied for functional relevance to NTD pathogenesis.

CONCLUSIONS

The candidate gene association approach can identify genes associated with human NTD risk when sufficiently large samples are tested, and appropriate statistical methods are used to adjust for confounding factors and multiple testing. Replication of positive findings in different populations remains useful to delineate common genetic risk factors for NTD. Evolving methodologies such as high-throughput genotyping and sequencing platforms will greatly facilitate the discovery of genetic cause of human NTD by screening the complete human genome using the GWAS approach. To reach the large sample sizes required for a GWAS approach, there need to be collaborations within the NTD research community.

Acknowledgments

Grant sponsor: National Institutes of Health; Grant numbers: P01-HD35946, R01-NS039818; Grant sponsor: Shriners Hospital for Children—project 8580.

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Table 1

List of Candidate Genes for Replication Study

Function	Gene Symbol	References
Folate, B12 transport	*SLC19A1/RFC1, CUBN*	De Marco et al., 2003; Pei et al., 2008; Franke et al., 2009
Folate metabolism	*DHFR, MTHFD1, MTHFD1L, MTHFR, TYMS*	Brody et al., 2002; Volcik et al., 2003; Martinez et al., 2009; Parle-McDermott et al., 2009; Shaw et al., 2009
Methionine cycle	*BHMT, MTRR*	Boyles et al., 2006; Franke et al., 2009; Shaw et al., 2009
Methyltransferases	*CHKA, ICMT, PCMT1, PRMT1*	Zhu et al., 2004; Enaw et al., 2007; Franke et al., 2009
Transsulfuration	*CBS, CTH*	Franke et al., 2009; Martinez et al., 2009; Shaw et al., 2009
Glucose metabolism	*GLUT1, HK1, LEPR, UCP2*	Volcik et al., 2003; Davidson et al., 2008; Mitchell et al., 2009
Nucleic acid repair	*APEX1, XPD*	Olshan et al., 2005
Cell recognition	*NCAM1*	Deak et al., 2005
Animal model and embryogenesis	*PDGFRA, T* (*Brachyury*), *TP53*	Shields et al., 2000; Joosten et al., 2001; Zhu et al., 2004; Pangilian et al., 2008