Neural tube defects (NTDs) are common, severe congenital malformations whose causation involves multiple genes and environmental factors. Although more than 200 genes are known to cause NTDs in mice, there has been rather limited progress in delineating the molecular basis underlying most human NTDs. Numerous genetic studies have been carried out to investigate candidate genes in cohorts of patients, with particular reference to those that participate in folate one-carbon metabolism. Although the homocysteine remethylation gene MTHFR has emerged as a risk factor in some human populations, few other consistent findings have resulted from this approach. Similarly, attention focused on the human homologues of mouse NTD genes has contributed only limited positive findings to date, although an emerging association between genes of the non-canonical Wnt (planar cell polarity) pathway and NTDs provides candidates for future studies. Priorities for the next phase of this research include: (i) larger studies that are sufficiently powered to detect significant associations with relatively minor risk factors; (ii) analysis of multiple candidate genes in groups of well-genotyped individuals to detect possible gene–gene interactions; (iii) use of high throughput genomic technology to evaluate the role of copy number variants and to detect ‘private’ and regulatory mutations, neither of which have been studied to date; (iv) detailed analysis of patient samples stratified by phenotype to enable, for example, hypothesis-driven testing of candidates genes in groups of NTDs with specific defects of folate metabolism, or in groups of fetuses with well-defined phenotypes such as craniorachischisis.
Although the causative genes are yet to be identified, these studies may result in identification of candidate sequences that can be evaluated in larger populations. An alternative approach exploits the association of NTDs with chromosomal anomalies such as trisomies 13 and 18 (9), suggesting that gene-dosage can affect neural tube closure. Rearrangements involving deletions, duplications or balanced translocations are likely to be most informative, with fine mapping of chromosomal breakpoints enabling identification of specific loci (10).

In some studies, direct mutation screening of candidate genes has been carried out in cohorts of patients (11), but the vast majority involve statistical association analysis of sequence variants in or near candidate genes. Most work has involved case-control analysis, comparing the frequency of ‘risk’ alleles in affected individuals and/or mothers with a matched unaffected cohort. More sophisticated studies have used the transmission disequilibrium test (TDT) in family trios (mother, father and affected child), which is less dependent on population structure. In the remainder of this article, we review the main candidate gene studies which have arisen primarily from analysis of folate metabolic pathways and mouse models of NTDs. Boyles et al. (11) published a comprehensive review of this field up to 2004, and an updated candidate gene list is presented in Table 1.

**CANDIDATE GENES FROM FOLATE METABOLISM**

Epidemiological studies provide an opportunity to identify risk factors for NTDs, such as dietary or teratogenic agents, to which susceptibility may be modified by genetic predisposition (12–14). Among environmental factors, folate status plays a key role in determining NTD risk (15,16). Maternal supplementation with folic acid during pregnancy reduces NTD frequency (17,18) whereas reduced serum folate and/or elevated homocysteine (an inverse indicator of folate status) are observed in some mothers of NTD fetuses, and are considered risk factors for NTDs (19–21). However, NTDs are not simply a condition of folate deficiency: maternal folate levels in most human NTD-affected pregnancies are in the ‘normal’ range (22), suggesting that low folate status may increase susceptibility but is not directly causative. Similarly, in mice dietary folate deficiency can cause significant embryonic growth retardation but does not cause NTDs (16,23,24). Hence, sub-optimal folate status may pre-dispose to NTDs in combination with additional factors, either environmental or genetic.

The intricate interplay and cross-regulation between elements of one-carbon (folate) metabolism (Fig. 1) complicates the teasing out of events that impinge on neural tube closure. In mice, key cellular functions in the developing embryo include methylation reactions and biosynthesis of nucleotides that support rapid cellular proliferation (2,25). Cranial NTDs arise when the methylation cycle is inhibited (26,27), and in null embryos for DNA methyltransferase 3B (28). In contrast, exogenous homocysteine does not cause NTDs (29–31), even in genetically predisposed *splotch* embryos (24) and may be an indicator of impaired folate or methylation cycle activity.

If folate status interacts with genetic factors in the causation of NTDs, this could involve either folate-related or folate-independent genes. To date, most emphasis has been placed on the evaluation of folate-related genes as NTD candidates (32,33) (Table 1). Further support comes from analysis of primary cell lines obtained from NTD fetuses, which indicates that a genetically-determined abnormality of folate metabolism is present, in at least a proportion of cases (34). However, identifying specific NTD-predisposing genetic lesions has proven far from straightforward. Although a number of variants have been widely studied, inconsistent results between different cohorts and populations (Table 1) indicate that very few, if any, have a major causative effect. Below, we sub-divide the candidate folate-related genes into three functional categories.

**Methylation related genes**

Among folate-related genes, 5,10-methylene tetrahydrofolate reductase (MTHFR) has been the principal focus of attention, following reports that the 677C>T (A222V; rs1801133) polymorphism is associated with increased risk of NTDs in Dutch and Irish populations (35–37). Other populations show no association (38,39) or even a protective effect (40,41) (Table 1). A meta-analysis, including genotype data from 27 studies up to 2004, suggests that the 677TT genotype confers an overall 1.9 times increase in NTD risk (Odds ratio: 1.9; 95% confidence interval: 1.6–2.2) (15). A more recent meta analysis (42) found a positive association only in non-latin groups, principally the Irish population.

The action of MTHFR generates 5-methylTHF for remethylation of homocysteine, at the expense of other folates required for purine and pyrimidine biosynthesis (Fig. 1). The A222V variant protein has reduced function and is associated with elevated plasma homocysteine (36). Nullizygosity for MTHFR in mice also results in elevated homocysteine and diminished DNA methylation (43), although NTDs are not observed under either normal or folate-deficient conditions. Moreover, MTHFR nullizygosity does not exacerbate the folate-responsive *splotch* mutation (43–45). These data suggest that in populations where MTHFR is a risk factor, additional interacting factors are likely to be present.

The link between reduced methylation/elevated homocysteine and NTDs has prompted analysis of variants in other genes that could influence the methylation cycle through remethylation (MTR, MTRR, BHMT and BHMT2) or transulfuration (CBS) of homocysteine (11) (Table 1). In general, mildly elevated risks have been identified in some studies but rarely replicated. MTRR (methionine synthase reductase) functions to maintain activity of MTR (methionine synthase), and a variant form (I22M, encoded by 66A>G) was reported as an NTD case and maternal risk factor in some studies, but not others (Table 1). Mouse studies do not support a role for these genes in NTDs: targeted deletion of *Mtr* is embryonic lethal prior to neurulation stages and heterozygotes do not show NTDs (46). Similarly, reduced activity of *Mtrr* and loss of *cbs* function do not cause NTDs, although elevated plasma homocysteine is observed (47,48).
| Human gene | Type of candidate | Reference | Population studied | Sample size | Type of study | Summary of results/conclusion |
|------------|-------------------|-----------|--------------------|-------------|--------------|--------------------------------|
| AHCY (S-adenosylhomocysteine hydrolase) | One carbon metabolism | (56) Dutch | 180 SB patients, 190 controls | Case–control study | No association |
| ALDH1L1 (Aldehyde dehydrogenase 1, member L1) | One carbon metabolism | (56) Dutch | 180 SB patients, 190 controls | Case–control study | Nominally significant association with Asp793Glu variant |
| ALDH1A2 (Retinaldehyde dehydrogenase Type 2, RALDH2) | Retinol metabolism | (104) USA | 318 SB families | Family based association study | One polymorphism associated with increased SB risk (tentative association for two others) |
| AMD1 (Adenosyl methionine decarboxylase 1) | One carbon metabolism | (56) Dutch | 180 SB patients, 190 controls | Case–control study | No association |
| APE1 (apurinic endonuclease1) | DNA repair | (112) Mixed USA | 380 SB cases, 350 controls | Case–control study | No association |
| BHMT (betaine-homocysteine methyltransferase) | One carbon metabolism | (113) Mixed USA | 252 SB cases, 337 controls | Case–control study | No association |
| BHMT2 (betaine-homocysteine methyltransferase 2) | One carbon metabolism | (103) Dutch | 259 SB cases, 359 controls | Case–control study | No association |
| BRCA1 (breast cancer 1) | NTDs in mouse mutant | (114) USA | 268 SB patients and parents | Case–control study | No association |
| CAT (catalase) | Oxidative stress | (115) Mixed USA | 507 SB cases, 185 controls | Case–control study | No association |
| CBS (cystathionine beta-synthase) | Folate metabolism | (116) UK | 207 NTD cases (200 mothers, 93 fathers), 601 controls, 542 control mothers | Case–control study | No association for 844ins68 |
| CFL1 (n-cofilin) | NTDs in mouse mutant | (117) Mixed USA | 246 SB cases, 336 controls | Case–control study | No association |
| CHKA (choline kinase A) | One carbon metabolism | (118) Mixed USA | 103 SB cases, 338 controls | Case–control study | No association |
| CITED2 | NTDs in mouse mutant | (119) Mixed USA | 64 SB cases, 72 controls | Mutation screen and case–control | No mutations. No association of three 5'-UTR SNPs with risk |
| COQ3 (Coenzyme Q3 homolog, methyltransferase) | Methylation | (56) Dutch | 180 SB patients, 190 controls | Case–control study | No association |
| CRABPI (cellular retinoic acid binding protein I) | Retinol metabolism | (104) USA | 230 SB cases, 318 SB families | Mutation screen and family based association study | No mutations. No association (3 SNPs tested) |
| CRABPII (cellular retinoic acid binding protein II) | Retinol metabolism | (104) USA | 230 SB cases, 318 SB families | Mutation screen | No mutations. No association |
| CTH (Cystathionase) | One carbon metabolism | (104) Dutch | 230 SB cases, 318 SB families | Mutation screen | No mutations. No association (3 SNPs tested) |
| CUBN (cubulin) | Endocytosis (folate transport) | (56) Dutch | 179 SB patients, 190 controls | Case–control study | No association |
| CYP26A1 (cytochrome P450) | Retinol metabolism | (104) USA | 230 SB cases, 318 SB families | Mutation screen | No mutations. No association |
| CYP26B1 (cytochrome P450) | Retinol metabolism | (104) USA | 230 SB cases, 318 SB families | Mutation screen | No mutations. No association (5 SNPs tested) |
Table 1. Continued

| Human gene          | Type of candidate                  | Reference | Population studied | Sample size | Type of study       | Summary of results/conclusion                                                                 |
|---------------------|------------------------------------|-----------|--------------------|-------------|---------------------|------------------------------------------------------------------------------------------------|
| DHFR (Dihydrofolate reductase) | Folate metabolism               | (120)     | Mixed USA          | 61 SB cases and parents (multi-affected families) and 219 controls | Case–control study of 19-bp intron-1 deletion | The del/del genotype was more frequent in mothers of SB cases, compared with controls. No association in fathers or patients 19-bp Intron deletion shows protective effect. May increase mRNA levels |
|                     |                                    | (121)     | Irish              | 283 cases (and 280 mothers, 279 fathers and 256 controls. SB (95%) or encephalocele (5%) | Case–control study of 19-bp deletion and two 3′-UTR variants. | 19-bp Intron deletion not associated with NTDs. No effect on expression  |
|                     |                                    | (122)     | Dutch              | 109 patients, 121 mothers (SB), 234 paediatric controls, 292 control women | Case–control study of 19-bp deletion and 9-bp repeat in 5′-UTR | 19-bp Intron deletion not tested. Intron deletion not tested |
|                     |                                    | (56)      | mixed US           | 180 SB patients, 190 controls 259 SB cases, 359 controls | Case–control study | No association |
|                     |                                    | (103)     |                     | 126 SB (open); 103 SB (closed); 49 anencephalic; 192 controls | Case–control study | No association |
|                     |                                    | (41)      | Mixed UK           | 180 SB patients, 190 controls 259 SB cases, 359 controls | Case–control study | No association |
|                     |                                    |           |                    | 126 SB (open); 103 SB (closed); 49 anencephalic; 192 controls | Case–control study | No association |
|                     |                                    |           |                    | 180 SB patients, 190 controls 259 SB cases, 359 controls | Case–control study | No association |
|                     |                                    |           |                    | 180 SB patients, 190 controls 259 SB cases, 359 controls | Case–control study | No association |
|                     |                                    |           |                    | 180 SB patients, 190 controls 259 SB cases, 359 controls | Case–control study | No association |
| FOLR1 (Folate receptor 1) | Folate transport               | (56)      | Dutch              | 180 SB patients, 190 controls 259 SB cases, 359 controls | Case–control study | No association |
| FOLR2 (Folate receptor 2) | Folate transport               | (56)      | Dutch              | 180 SB patients, 190 controls 259 SB cases, 359 controls | Case–control study | No association |
| FOLR3 (Folate receptor 3) | Folate transport               | (56)      | Dutch              | 180 SB patients, 190 controls 259 SB cases, 359 controls | Case–control study | No association |
| FPGS (Folypolyglutamate synthase) | Cellular folate retention   | (56)      | Dutch              | 180 SB patients, 190 controls 259 SB cases, 359 controls | Case–control study | No association |
| FTCD (Formiminotransferase cyclodeaminase) | One carbon metabolism | (56)      | Dutch              | 180 SB patients, 190 controls 259 SB cases, 359 controls | Case–control study | No association |
| GAMT (Guanidinoacetate N-methyl transferase) | One carbon metabolism | (56)      | Dutch              | 180 SB patients, 190 controls 259 SB cases, 359 controls | Case–control study | No association |
| GAPD (glyceraldehyde 3 phosphate dehydrogenase) | Glucose metabolism         | (115)     | Mixed USA          | 180 SB patients, 190 controls 259 SB cases, 359 controls | Case–control study | No association |
| GART (Phosphoribosylglycinamide formyltransferase, phosphoribosyl glycaminide synthetase, phosphoribosyl aminomimidazole synthetase) | Purine biosynthesis (one carbon metabolism) | (56)      | Dutch              | 180 SB patients, 190 controls 259 SB cases, 359 controls | Association study | No association |
| GCPII (glutamate carboxypeptidase), FOLH1 (Folate hydrolase) | Folate metabolism            | (116)     | UK                 | 208 NTD cases (200 mothers, 92 fathers), 600 child, 531 mother controls | Case–control study | No association for 1561C>T |
| GGH (Gamma-glutamyl hydrolase) | Folate metabolism            | (56)      | Dutch              | 180 SB patients, 190 controls 259 SB cases, 359 controls | Case–control study | No association |
| GLUT1 (glucose transporter 1) | Glucose metabolism         | (56)      | Dutch              | 180 SB patients, 190 controls 259 SB cases, 359 controls | Case–control study | No association |
| GLUT4 (glucose transporter 4) | Glucose metabolism         | (115)     | Mixed USA          | 507 SB cases, 185 controls | Case–control study | No association |
| HK1 (hexokinase 1) | Glucose metabolism           | (115)     | Mixed USA          | 507 SB cases, 185 controls | Case–control study | No association |
| HK2 (hexokinase 2) | Glucose metabolism           | (115)     | Mixed USA          | 507 SB cases, 185 controls | Case–control study | No association |
| ICMT (Isoprenylcysteine carboxyl methyltransferase) | Protein methylation | (56)      | Dutch              | 180 SB patients, 190 controls 259 SB cases, 359 controls | Case–control study | No association |
| INS (insulin) | Glucose metabolism           | (115)     | Mixed USA          | 507 SB cases, 185 controls | Case–control study | No association |
| INSR (insulin receptor) | Glucose metabolism         | (115)     | Mixed USA          | 507 SB cases, 185 controls | Case–control study | No association |
| LEP (leptin) | Glucose metabolism           | (115)     | Mixed USA          | 507 SB cases, 185 controls | Case–control study | No association |
| LEPR (leptin receptor) | Glucose metabolism         | (115)     | Mixed USA          | 507 SB cases, 185 controls | Case–control study | No association |
| MAT1A (Methionine adenosyltransferase I, alpha) | One carbon metabolism | (56)      | Dutch              | 180 SB patients, 190 controls 259 SB cases, 359 controls | Case–control study | No association |
| Gene | Metabolism | Population | Study Type | Results |
|------|------------|-------------|------------|---------|
| MAT2A (Methionine adenosyltransferase II, alpha) | One carbon metabolism | Dutch | Case–control study | No association |
| MGMT (O-6-Methylguanine DNA methyltransferase) | DNA methylation | Dutch | Case–control study | No association |
| MTHFD1 (methylene tetrahydrofolate dehydrogenase/ methyltetrahydrofolate-cyclohydrolase/ formyltetrahydrofolate synthetase) | Folate metabolism | Irish | Case–control study | Maternal AA genotype confers increased risk to offspring |
| | | Italian | Case–control study | No association |
| | | Dutch | Case–control study | No association |
| MTHFD2 | Folate metabolism | Dutch | Case–control study | No association |
| MTHFR (5,10-methyltetrahydrofolate reductase) | Folate metabolism | UK | Case–control study | No association |
| | | Mixed USA | Case–control study | No association for 8 SNPs tested |
| | | Polish | Case–control study | No association for 677C>T. No association for 1298A>C. |
| | | Irish | Mutation screen. | Possible association with SB for variants tested. Unlike to be independent risk factors (association likely due to linkage disequilibrium with 677C>T) |
| | | Mixed USA | Family-based association study | No association for 677C>T |
| | | Italian | Case–control screen | T allele more frequent in cases than controls. A1298C not different between groups |
| | | Mexican | Case–control study | Maternal TT confers higher risk than CC for anencephaly. A1298C not associated with NTDs |
| | | Indian | Case–control for C677T and A1298C | 677T more frequent in mothers of cases than in controls (only for lower defects). No difference in A1298C frequency |
| Human gene | Type of candidate | Reference | Population studied | Sample size | Type of study | Summary of results/conclusion |
|------------|------------------|-----------|--------------------|-------------|---------------|-------------------------------|
|   | | (129) Mexican (Yucatan) | | 108 cases (97 SB, 4 anencephalic, 7 encephalocele), with 147 parents. 120 controls | Case–control, screen for A1298C | No association |
|   | | (130) French | | 77 NTD mothers. 61 controls | Case–control study | No association for 677C>T. Reduced risk for 1298A>C allele TT genotype less frequent in case mothers (but small numbers) |
|   | | (131) Chinese | | 38 mothers of NTD cases. 80 controls | Case–control to evaluate 677C>T | No association for 677C>T or 1298A>C |
|   | | (56) Dutch | | 180 SB patients, 190 controls | Case–control study | Increased risk, OR2.0, of SB associated with 677C>T Slight protective effect for open SB of 677TT genotype |
|   | | (103) Mixed USA | | 259 SB cases, 359 controls | Case–control (13 SNPs) | |
|   | | (41) Mixed UK | | 126 SB (open); 103 SB (closed); 49 anencephalic; 192 controls | Case–control for C677T and A1298C | |
|   | | (56) Dutch | | 180 SB patients, 190 controls | Case–control study | No association |
|   | | (103) Mixed USA | | 259 SB cases, 359 controls | Case–control study | No association |
|   | | (65) US | | 234 control children, 292 control women. | Case–control study | No association |
|   | | (116) UK | | 201 NTD cases (203 mothers, 88 fathers). 601 child, 532 mother controls | Case–control study | G allele associated with increased risk. Additional risk in combination with MTR 2756G allele |
|   | | (132) Irish | | 575 NTD families 95% SB). 487 controls. | Case–control and family-based analysis for three variants | No association for 66A>G (I22M) with SB risk (except possible paternal effect). No association for S175L or K350R |
|   | | (133) Dutch | | 109 cases (open SB) and parents. 234 control children, 292 control women. | Case–control screen for 66A>G | In this study and meta-analysis (including previous studies) maternal GG is associated with increased risk in offspring, but GG in child not associated with NTDs |
|   | | (130) French | | 77 NTD mothers. 61 controls | Case–control study | Marginally increased risk associated with 66G allele |
|   | | (56) Dutch | | 180 SB patients, 190 controls | Case–control study | No association |
|   | | (103) Mixed USA | | 259 SB cases, 359 controls | Case–control study | Modest increase in SB risk for three linked SNPs, but not 66A>G |
| Gene                        | Function                                      | Population | Sample Size | Study Type                      | Results                                                                 |
|-----------------------------|-----------------------------------------------|------------|-------------|---------------------------------|------------------------------------------------------------------------|
| MUT (methylmalonyl-CoA mutase) | One carbon metabolism                          | Mixed UK   | 126 SB (open); 103 SB (closed); 49 anencephalic; 192 controls | Case–control study                                                  | Marginal increased risk for combined NTDs                               |
| NAT1 (N-acetyltransferase 1)  | Folate metabolism and acetylation reactions   | Irish      | 279 NTD triads (mostly SB), 256 controls                  | Case–control and family-based study                                 | No association with NTDs for three variants tested                      |
|                             |                                               | Mixed USA  | 354 NTD families                                           | Family based association study                                      | 1095C>A not associated with SB risk (except in combination with maternal smoking) |
|                             |                                               | Mixed USA  | 374 NTD families                                           | Family based association study                                      | Composite genotype (6 SNPs) related to reduced function associated with lower SB risk for cases and mothers |
| NAT2 (N-acetyltransferase 2)  | Methylation reactions                         | Dutch      | 180 SB patients, 190 controls                             | Case–control study                                                  | No association between NNMT variants and SB risk                        |
| NNMT (Nicotinamide N-methyltransferase) | Methylation reactions                        | Mixed USA  | 252 SB cases, 335 controls                               | Case–control study                                                  | No association                                                         |
| NCAM1 (neural cell adhesion molecule1) | Embryonic cell adhesion                      | Dutch      | 180 SB patients, 190 controls                             | Association study                                                   | Risk of SB associated with intronic SNP (of 11 tested) in first 132 families, not in further 72 families |
| NOS1 (nitric oxide synthase 1)  | Possible effect on one carbon metabolism      | Dutch      | 180 SB patients, 190 controls                             | Case–control study                                                  | No association for repeat polymorphism                                  |
| NOS2 (nitric oxide synthase 2A)   |                                               | Dutch      | 180 SB patients, 190 controls                             | Case–control study                                                  | No association for SNPs and repeat polymorphism                         |
| NOS3 (nitric oxide synthase 3 endothelial) |                                       | Mixed US   | 301 families with SB                                      | Family based association study                                      | No association by TDT analysis. Maternal 894G>T associated with SB risk by log-linear modelling |
| hOGG1 (8-hydroxyguanine DNA-glycosylase1) | DNA repair                                    | Mixed USA  | 259 SB cases, 359 controls                               | Case–control study                                                  | 894G>T not independent risk factor. Possible risk interaction with MTHFR 677TTT |
| PAX3                        | NTDs in mouse mutant                          | Mixed USA  | 380 SB cases, 350 controls                               | Case–control study                                                  | No association                                                         |
| PCMT1 (l-isoaspartate O-methyltransferase) | Methylation reactions                      | Mixed USA  | 74 SB cases, 87 controls                                  | Mutation screen and case–control study                              | rs16863657 and related haplotype associated with increased SB risk      |
| PDGFRα (Platelet derived growth factor receptor alpha) | NTDs in Patch mouse mutant                  | Dutch      | 180 SB patients, 190 controls                             | Case–control study                                                  | Val/Val genotype associated with possible reduction in SB risk         |
|                             |                                               | US Hispanic| 43 NTD cases, 122 NTD mothers. 124 control infants, 127 control mothers | Case–control study                                                  | No association for Ile120Val PI promoter haplotypes with lower activity may be associated with maternal risk. Case numbers too small for conclusion |
|                             |                                               | Mixed USA  | 407 parent-child triads (SB), 164 controls                | Case–control and family based association (TDT)                     | No association for PI promoter haplotypes                              |
|                             |                                               | Dutch      | 88 SB cases, 56 SB mothers. 74 controls, 72 control mothers | Evaluated H1 and H2 promoter haplotypes                             | H1 promoter may be more frequent in cases than controls. Suggestion that BMI, glucose and inositol differentially interact with H1/H2 |
| PEMT (Phosphatidylethanolamine N-methyltransferase) | One carbon and choline metabolism            | Mixed USA  | 360 SB cases, 595 controls                               | Case–control study                                                  | No association with SB for two non-synonymous SNPs                     |

Continued
| Human gene | Type of candidate | Reference | Population studied | Sample size | Type of study | Summary of results/conclusion |
|-----------|------------------|-----------|--------------------|-------------|--------------|------------------------------|
| **PRKACA, PRKACB** (cAMP-dependent protein kinase A catalytic subunits) | NTDs in mouse mutants | (145) Mixed USA | 207 SB cases, 209 controls | Mutation screen and case–control study | No mutation. No association |
| **PRMT1** (Protein arginine methyltransferase 1) | Methylation reactions | (56) Dutch | 103 SB cases, 338 controls | Case–control study | Weak association with SB risk for 1 of 2 SNPs studied |
| **PRMT2** (Protein arginine methyltransferase 2) | Methylation reactions | (56) Dutch | 180 SB patients, 190 controls | Case–control study | No association |
| **PYCT1A** (CTP:phosphocholine cytidylyltransferase) | One carbon metabolism | (118) Mixed USA | 207 SB cases, 209 controls | Mutation screen and case–control study | No mutation. No association |
| **RNMT** (RNA (guanine-7-) methyltransferase) | Methylation reactions | (56) Dutch | 103 SB cases, 338 controls | Case–control study | No association |
| **SARDH** (Sarcosine dehydrogenase) | One carbon metabolism | (146) UK | 97 NTD mothers, 190 controls | Case–control study | Nominally significant association with two synonymous SNPs* |
| **SHMT** (Serine hydroxyl methyltransferase) | One carbon metabolism | (56) Dutch | 180 SB patients, 190 controls | Case–control study | No association for 80G>A (H27R) |
| **SLCA19A1, RFC-1** (reduced folate carrier), | Folate transport | (125) Mixed USA | 350 cases, 328 mothers, 245 fathers, 167 siblings. | Case–control study | No association for 80G>A |
| | | (147) Irish | 636 NTD families, 385 controls | Family-based association study | No association for 80G>A. 61-bp polymorphism under-represented in cases |
| | | (131) Chinese | 38 mothers of cases. 80 controls | Case–control study | Association of GG genotype with risk of NTD offspring |
| | | (148) Chinese | 104 NTD families, 100 control families | Case–control study | Elevated risk for GG genotype of cases or mother (if folic acid not used) |
| | | (56) Dutch | 180 SB patients, 190 controls | Case–control study | Nominally significant association for 80AA genotype and reduced risk* |
| | | (103) Mixed USA | 259 SB cases, 359 controls | Case–control study | No association with risk of SB for 6 SNPs tested. 80G>A not tested |
| | | (41) Mixed UK | 126 SB (open); 103 SB (closed); 49 anencephalic; 192 controls | Case–control study | No association |
| **SOD2** (superoxide dismutase 2) | Oxidative stress | (115) Mixed USA | 507 SB cases, 185 controls | Case–control study | No association |
| | | (149) Mixed USA | 316 SB families | Family-based association study | No association for 776C>G A2 promoter allele more frequent in cases than expected |
| | | | | Case–control study | No association with SB risk for 6 SNPs |
| **TCNII** (transcobalamin II) | One carbon metabolism | (150) Irish | ~350 NTD families, ~700 controls. | Case–control study | No association for 776C>G |
| | | | | Family-based association study | No association for Arg259Pro |
| | | | | Case–control study | Two non-coding variants associated with case NTD risk and two variants with maternal risk (but no multiple testing correction) |
| | | | | Family-based association study | No association for one SNP* |
| **TXN2** (thioredoxin2) | NTDs (SB) in mouse knockout | (130) French | 77 NTD mothers. 61 controls | Case–control study | No association for 776C>G |
| | | (56) Dutch | 180 SB patients, 190 controls | Case–control study | No association for Arg259Pro |
| | | | | Case–control study | Two non-coding variants associated with case NTD risk and two variants with maternal risk (but no multiple testing correction) |
| **TP53** (p53) | NTDs in mouse mutant | (152) Irish | 549 NTD cases, 532 mothers, 481 fathers. 999 controls | Case–control study | No association |
| | | | | Family based association studies | Nominally significant association for one SNP* |
| **TRDMT1** (tRNA aspartic acid methyltransferase 1) | One carbon metabolism | (115) Mixed USA | 507 SB cases, 185 controls | Case–control study | One SNP* |
| | | (56) Dutch | 180 SB patients, 190 controls | Family-based association study | No association for one SNP* |
| Gene          | Function                          | Study Details                     | Results                                                                 |
|--------------|-----------------------------------|-----------------------------------|-------------------------------------------------------------------------|
| *TYMS*       | Folate metabolism and pyrimidine biosynthesis | Mixed USA, 264 SB cases, 259 controls. | Mutation screen and case–control for 28-bp repeat in 5′-UTR and 6-bp deletion in 3′-UTR. No association for *TYMS*. 3′-UTR polymorphism associated with increased SB risk in non-Hispanic white population. Further increased risk with 5′-UTR polymorphism. |
| *UCP2*       | Energy metabolism Previous study indicates association with NTDs | UK, 197 NTD cases, 194 mothers, 93 fathers, 179 control infants, 177 control mothers. | Case–control study of 28-bp repeat. No association. Modest increase in SB risk for three linked SNPs (of 5 tested). |
| *VANGL1*     | PCP gene homologue; Parologue of *VANGL2* | Mixed UK and USA, 66 (21 craniorachischisis, 24 SB, 21 anencephalic) and 200 controls. | Mutation screen. No causative mutations. One missense variant, present in controls. |
| *VANGL2*     | Mouse model: Craniorachischisis in loop-tail mice | Italian and French, 144 (80 SB, 7 craniorachischisis, 22 closed spinal dysraphism, 35 caudal regression). 172 Italian and CEPH controls. | Mutation screen. Three missense mutations (two in myelomeningocele, R274Q and M328T; 1 in caudal regression, V239I). V239I has functional effect on interaction with Dvl proteins. Ten missense variants in 13 individuals (absent in 1187–1462 controls). Five in highly conserved residues (two in myelomeningocele, three in closed spinal dysraphism/caudal regression syndrome) |
| *XPD*        | DNA repair                         | Mixed USA, 380 SB cases, 350 controls. | Case–control study. Mildly elevated risk associated with 571Gln. |
| *XRCC1*      | DNA repair                         | Mixed USA, 380 SB cases, 350 controls. | Case–control study. No association. |
| *XRCC3*      | DNA repair                         | Mixed USA, 380 SB cases, 350 controls. | Case–control study. No association. |
| *ZIC1*       | Brain defects in mouse mutant     | Dutch, 117 NTDs (SB, Anencephaly, Encephalocele), 364 controls. | Mutation screen. No mutations. |
| *ZIC2*       | NTDs in mouse mutant              | Dutch, 117 NTDs (SB, Anencephaly, Encephalocele), 364 controls. | Mutation screen; case–control study. Alanine deletion in one patient. Frequent polymorphism (1059C>T) has no association. One silent variant (858G>A) in one patient. |
| *ZIC3*       | NTDs in mouse mutant              | Dutch, 117 NTDs (SB, Anencephaly, Encephalocele), 364 controls. | Mutual screen. One missense variant (858G>A) in one patient. |

*Nominally significant association which does not stand after correction for multiple testing. SB, defined as spina bifida (myelomeningocele) in study criteria. For studies labelled NTDs, populations were mixed (multiple types of NTDs) or undefined.*
Folate cycle enzymes required for nucleotide biosynthesis

*MTHFD1* encodes the cytoplasmic trifunctional C1THF synthase enzyme. A polymorphism (1958G>A; rs2236225) which results in an R653Q substitution in the 10-formylTHF synthetase domain was found to be both a maternal and NTD case risk factor, in the Irish and Italian populations (49–51), although not in the Dutch (51,52) or British (41). The R653Q polymorphism causes reduced C1THF synthase activity in cell lines, resulting in diminished purine biosynthesis (53). A promoter polymorphism (rs1076991C>T) in *MTHFD1*, that reduces transcriptional activity *in vitro*, was also associated with NTD case and maternal risk, in combination with R653Q (54).

Folate transport

Another attractive group of candidate genes are those encoding proteins required for transport, uptake and cellular retention of folates. This includes folate receptors *FRα* (*Folr1* in mice), *FRβ* and *FRγ*, *RFC1* (reduced folate carrier), *GCPII* (follyl-γ-glutamate carboxypeptidase) and *FPGS* (follypolyglutamate synthetase) (32,33). Increased risks associated with variants in *RFC1* and *GCPII* are not reproduced in all studies (Table 1), although the recently identified proton-coupled folate transporter *PCFT* (SLC46a1) is not required for embryonic survival or neural tube closure in mice (55), but has not yet been investigated in humans. A recent case–control study revealed a possible association with reduced risk of spina bifida for a polymorphism in *CUBN* (Cubulin), which encodes a membrane-associated multi-ligand endocytic receptor expressed in the neural folds and yolk sac (56). Together, cubulin and its partner protein megalin are involved in binding and endocytic uptake of a large number of different proteins, several of which could be important for neural tube closure, including the intrinsic factor-cobalamin complex (IF-B12) and folate binding protein (folate receptor) (57,58). Intriguingly, *Cubn* was one of the most up-regulated genes in a microarray analysis of *Rfc1* null mouse embryos (59), which may
reflect a compensatory mechanism to enhance endocytic folate uptake via Folr1. Hence, CUBN merits further attention as a potential risk factor, especially in conjunction with RFC1.

In view of the apparent resilience of mouse neurulation to specific genetic disturbance of the methylation cycle, analysis of compound mutants with other folate-related or NTD susceptibility alleles would be of considerable interest. In our analysis of NTD cell lines, impaired folate cycle activity did not correlate with known variants in MTHFR, MTHFD1, DHFR, GCPII, MTR, MTRR or RFC1 (34), encouraging the view that currently unknown genetic influences on folate metabolism remain to be identified in many NTD cases.

CANDIDATE GENES FROM THE MOUSE

The potential complexity of NTD genetics is illustrated by the fact that 200 or more different mouse genes result in NTD phenotypes either through naturally occurring, induced or targeted mutations (2,25). Many of the NTD-causing mouse mutations implicate specific signalling pathways such as non-canonical Wnt signalling (see below), maintenance of the cell cycle, regulation of the actin cytoskeleton, chromatin organization or epigenetic modifications including methylation and acetylation. Recently, NTDs were observed in mice null for Mib2 (60), Smurf1/2 (61) and Hectd1 (62), which all encode E3 ubiquitin ligases, suggesting a possible role in neurulation for protein ubiquitination and targeted degradation. The human homologues of some of these mouse NTD genes have been examined in case–control association studies or directly sequenced in mutation screens, although with very few significant findings to date (Table 1).

It is important to ask how appropriate are the mouse models as paradigms for human NTDs? At the embryonic level, the events of neurulation appear extremely similar between mice and humans. For example, the initial fusion event, Closure 1, occurs at a closely similar stage and body axial level in both species, as does initiation of closure in the forebrain (Closure 3) and completion of spinal closure at the posterior neuropore. One point of variation concerns de novo initiation of closure at the forebrain/midbrain boundary (Closure 2 in mice) which may be absent from human neurulation (63). Hence, brain closure could be a rather simpler process in humans than mice.

Another potential difference between mouse models and human NTDs is that many gene-specific homozygous null mouse embryos exhibit phenotypes additional to NTDs, such as prenatally lethal heart defects. Such syndromic examples do not appear particularly close models for human NTDs, which are primarily non-syndromic (64). On the other hand, detailed analysis of a few of the mouse mutants suggests that isolated NTDs can also result from the effect of hypomorphic alleles, combinations of heterozygous mutations, genetic background effects and/or gene–environment interactions. This partial loss of function or multifactorial aetiologies may more closely resemble human NTDs. For example, NTDs in splotch mice result from homozygosity for mutations in Pax3 (23,65) but can also occur, or be exacerbated, as a result of interaction with mutations in other genes including neurofibromin1 (66) and grainyhead-like-3 (67). Environmental factors including folate deficiency and arsenic can exacerbate NTDs in homozygous splotch mutants, or induce NTDs in the usually unaffected heterozygotes (24,68). Although association studies in humans have provided little evidence to implicate PAX3 mutations in human NTDs (69,70), the possible contribution of gene–gene and gene–environment interactions indicates that larger scale studies may be needed before a role for PAX3 in human NTDs can be completely ruled out.

The curly tail mouse also exhibits features typical of the multifactorial aetiology of human NTDs (71). Spinal NTDs are partially penetrant in homozygous ct/ct mutant embryos, with the frequency of defects strongly affected by genetic modifiers (72). The major ct gene is a hypomorphic allele of Grs1h3, whose knockouts display completely penetrant spina bifida (73–75). The ct mutation appears to affect a regulatory region, emphasising the need for consideration of possible non-coding mutations in human NTDs. Moreover, there is a strong effect of environmental factors, including a protective effect of supplemental inositol (76). A key role for inositol in neural tube closure is supported by the finding that inositol deficiency in vitro causes NTDs (77), inositol may prevent diabetes-associated NTDs (78) and the recent finding of NTDs in embryos carrying a hypomorphic allele of inositol 1,3,4-trisphosphate 5/6-kinase (Ipk1), a key enzyme in inositol phosphate metabolism (79).

Figure 2. Diagrammatic representation of non-canonical Wnt signalling in a mammalian cell. Black arrows indicate the signalling pathway necessary for establishment of planar cell polarity (PCP). Known biochemical interactions are indicated by blue arrows and genetic interactions are shown by red arrows. Ankdr6 is the mammalian homologue of Diego which in Drosophila interacts with Fz, Vang and Pk, but has not been studied in vertebrates.

Planar cell polarity signalling and NTDs

A major advance in understanding the genetic basis of neurulation has been the finding that initiation of closure at the hindbrain-cervical boundary (Closure 1) requires non-canonical Wnt signalling: the so-called planar cell polarity (PCP) pathway (Fig. 2). PCP signalling was defined originally...
in *Drosophila*, as a genetic cascade involving the transmembrane receptor frizzled and the cytoplasmic protein dishevelled, but without a requirement for β-catenin (80–83). This pathway is required to specify planar polarity in epithelia including the wing and compound eye. In vertebrates, non-canonical Wnt signalling is highly conserved, underpinning tissue and cellular polarity during morphogenesis in systems as diverse as gastrulation and the coordinated orientation of stereociliary bundles in inner ear hair cells (84–90).

A potential role for PCP in NTDs first came to light following positional cloning of *Vangl2* (the homologue of *Drosophila strabismus/Van gogh*) in the *loop-tail* mouse mutant which exhibits the severe NTD, craniorachischisis (91,92). Subsequently, the same NTD phenotype was found in other mouse mutants and targeted gene knockouts (Table 2) almost all of which have been implicated biochemically in the PCP pathway (*e.g.* Celsr1, *Dvl*) or which interact genetically with recognized PCP genes (*e.g.* *Scrb*, *Ptk7*) (93,94). Interestingly, the double knockout for *Smurf1* and *Smurf2* was recently found to display craniorachischisis and other characteristic PCP defects. These genes encode ubiquitin ligases whose targets include Prickle1 (Fig. 2), supporting the crucial nature of PCP signalling for initiation of neural tube closure (61).

In view of these findings in mice, PCP genes emerge as excellent candidates for causation of craniorachischisis in humans. Nevertheless, sequence analysis has so far failed to identify mutations in human *VANGL2* or its parologue *VANGL1* in a group of patients with craniorachischisis (95,96). Reports of other PCP gene analysis in similar patients are awaited. Although craniorachischisis is the obvious NTD phenotype for study, the *VANGL* genes have also been analysed in patients with anencephaly and open and closed spina bifida. No mutations were reported in *VANGL2* (95,96) but several highly conserved and unique, heterozygous missense variants were identified in *VANGL1* in patients with either myelomeningocele or closed spina bifida, as well as caudal regression syndrome (96,97). To date a functional effect has been demonstrated for one of these putative mutations, where V239I (identified in caudal regression syndrome) results in loss of interaction between VANGL1 and DVL proteins (96).

Interestingly, loss of *Vangl1* function is insufficient to cause NTDs in mice, although compound heterozygotes with *Vangl2* (*loop-tail*) develop craniorachischisis (98). Nevertheless, there is increasing evidence that PCP genes can contribute to NTDs other than craniorachischisis (Table 2). For example, double heterozygotes carrying both *Vangl2* and *Ptk7* develop spina bifida (94) although *Vangl2* double mutants with *cordon-bleu* (*Cbleu*) or *Cthrcl* develop exencephaly (99,100). In contrast, *Vangl2:Scrb* and *Vangl2:Dvl3* double heterozygotes develop craniorachischisis (101,102). It remains to be determined why *Vangl2* displays this variable phenotypic behaviour when combined with different PCP and other mutants. Hence, although non-canonical Wnt signalling has been firmly linked with Closure 1 in mice, it is possible that genes in this pathway play more diverse roles in human neural tube closure.

**CONCLUSIONS**

The identification of genetic risk factors for human NTDs is complicated by the multiplicity of genes participating
in neurulation, and the importance of gene–environment interactions. Sequence analysis of candidate genes implicated from their role in mouse models has revealed putative mutations in a few genes, but each in only a small number of patients. Association studies of common polymorphic variants, particularly related to folate one-carbon metabolism, indicate risk factors such as MTHFR. However, no specific folate-related gene has yet been implicated as a major determinant of risk for NTDs. Large-scale studies will be required to provide sufficient statistical power to convincingly test whether such variants are truly NTD susceptibility factors (56,103). It will also be essential, to evaluate multiple genes (folate-related and others) in the same individuals in order to detect possible compounding effects of combinations of risk alleles that, individually, might not be statistically significant (11,39). To date, very few studies have been sufficiently large to overcome issues of multiple testing bias in screening for gene–gene interactions (39,56,104). Examination of specific hypotheses may be fruitful where fewer NTD cases are available, particularly if combined with stratified sample sets in which cases are sub-divided on the basis of phenotype. For example, NTDs with abnormal folate metabolism have enabled a combined analysis of MTR and MTRR (105), and fetuses with craniorachischisis provide a focus for determining the role of PCP genes. Gene–environment interactions appear likely to contribute to NTD predisposition, with examples including interactions of MTHFR with multivitamin use (106), MTRR with vitamin B12 (107) and PDGFRA with inositol and zinc (108).

One limitation of the association studies of multiple folate-related candidate genes in NTDs is the predominant focus on known polymorphisms. In future, it will be necessary also to consider the possible existence of ‘private’ disease-causing mutations. Moreover, the potential for deleterious gene expression changes resulting from promoter mutations or copy number variation has been addressed in relatively few studies (10,108–110). Emerging technologies for high throughput sequencing and analysis of genomic deletions and copy number variations (111) offer the prospect, in the coming years, of progress in identification of candidate genes and screening for novel mutations in human NTDs.

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