Published in final edited form as:
J Med Genet. 2010 October ; 47(10): 677–685. doi:10.1136/jmg.2009.073775.

Transcobalamin II Receptor Polymorphisms Are Associated with Increased Risk for Neural Tube Defects

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Abstract

Objective: Women who have low cobalamin (vitamin B12) levels are at increased risk for having children with neural tube defects (NTDs). The transcobalamin II receptor (*TCblR*) mediates uptake of cobalamin into cells. We evaluated inherited variants in the *TCblR* gene as NTD risk factors.

Methods: Case-control and family-based tests of association were used to screen common variation in *TCblR* as genetic risk factors for NTDs in a large Irish group. A confirmatory group of NTD triads was used to test positive findings.

Results: We found two tightly linked variants associated with NTDs in a recessive model: *TCblR* rs2336573 (G220R) (p_{corr}=0.0080, corrected for multiple hypothesis testing) and *TCblR* rs9426 (p_{corr} =0. 0279). These variants were also associated with NTDs in a family-based test prior to multiple test correction (log-linear analysis of a recessive model: rs2336573 (G220R) (RR=6.59, p=0.0037) and rs9426 (RR=6.71, p=0.0035)). We describe a copy number variant (CNV) distal to *TCblR* and two previously unreported exonic insertion-deletion polymorphisms.

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Competing interests: None.

Conclusions: *TCblR* rs2336573 (G220R) and *TCblR* rs9426 represent a significant risk factor in NTD cases in the Irish population. The homozygous risk genotype was not detected in nearly one thousand controls, indicating this NTD risk factor may be of low frequency and high penetrance. Nine other variants are in perfect LD with the associated SNPs. Additional work is required to identify the disease-causing variant. Our data suggest that variation in *TCblR* plays a role in NTD risk and that these variants may modulate cobalamin metabolism.

Keywords

neural tube defects; spina bifida; transcobalamin II receptor (*TCblR*); cobalamin; vitamin B12; copy number variant (CNV)

INTRODUCTION

Neural tube defects (NTDs) are common birth defects, affecting ~1 in 1000 pregnancies [1, 2]. The neural tube closes during the fourth week of embryogenesis and gives rise to the spinal cord and brain. Incomplete closure of the neural tube causes a range of birth defects including spina bifida and anencephaly.

NTD etiology is multifactorial and includes environmental and genetic factors [3, 4]. Periconceptional folic acid supplementation in mothers can reduce the risk of an NTD affected pregnancy by up to 70% [5, 6]. Genetic variants relating to folate metabolism have also been implicated in contributing to NTD risk. The most well studied of these is the 677C>T variant in the methylene tetrahydrofolate reductase (*MTHFR*) gene (reviewed in [7]). In the European population, this variant may account for 26% of NTDs [8].

Cobalamin (vitamin B12) plays an important role in folate metabolism as a cofactor for methionine synthase (*MTR*), the enzyme that catalyzes the transfer of the methyl group from 5-methyl tetrahydrofolate (5-methyl THF) to homocysteine to produce methionine. Compromised methionine synthase function results in functional intracellular folate deficiency and a deficit of methyl groups for methylation reactions. Low maternal blood [9, 10, 11, 12, 13, 14, 15, 16, 17] or amniotic fluid [18, 19, 20, 21, 22] cobalamin levels have been associated with NTD risk. Levels of the cobalamin-transcobalamin II complex (holoTC) are also reduced in mothers during [23] or after [24] an NTD pregnancy.

Polymorphisms in cobalamin-related genes have been investigated for NTD risk. Several transporter proteins, receptors and converting enzymes are required to ensure that dietary cobalamin is delivered in an active form to methionine synthase and methylmalonyl-CoA mutase (*MUT*). Transcobalamin II (*TCN2*) transports cobalamin from the intestinal circulation into the bloodstream and ultimately to target tissues [25]. *TCN2* P259R (rs1801198) was reported to be a maternal NTD risk factor [26] although other studies examined multiple *TCN2* variants without detecting an effect [24, 27]. Methionine synthase reductase (*MTRR*) re-reduces oxidized cobalamin, thereby maintaining methionine synthase activity. Although some studies found no association between NTDs and variants in *MTR* or *MTRR* [28, 29, 30, 31, 32], others observed independent and/or joint effects [16, 33, 34, 35, 36, 37]. Cubilin (*CUBN*) is a receptor found on the luminal epithelium of the intestine and

kidney that binds the cobalamin-intrinsic factor complex. An intronic single nucleotide polymorphism (SNP, rs1907362) in *CUBN* has been associated with NTDs [38].

Uptake of the cobalamin-transcobalamin II complex into cells and tissues occurs via binding to the recently identified transcobalamin II receptor (*TCblR*, also known as *CD320* and *8D6*) [39]. Because of *TCblR's* essential role in cobalamin bioavailability, we evaluated its genetic variation for association with NTDs in a large Irish cohort. We report a significantly associated genetic risk factor for NTDs in *TCblR*.

MATERIALS AND METHODS

Study Populations

Recruitment of our Irish case and control populations has been previously described [40, 41, 42]. Our complete Irish case population consists of 586 families with an NTD affected child. This includes 442 full family triads (case, mother, father), 57 case-mother pairs, 4 case-father pairs, 44 cases-only and 39 families with parents or single parent only. In total there are 530 spina bifida cases and 21 encephalocele cases. Our control samples [n=999] were randomly selected from a bank of 56,049 blood samples taken from women during their first prenatal visit at the three major maternity hospitals in Dublin between 1986 to 1990, after excluding samples from women whose current or past pregnancies involved an NTD.

An additional 367 case families were recruited in the United Kingdom between 2001 and 2003, with the assistance of the UK Association for Spina Bifida and Hydrocephalus (ASBAH). This cohort includes 258 full family triads, 66 case-mother pairs, 2 case-father pairs, 38 case-only samples and 3 families with parents only. There are 354 spina bifida cases, 8 encephalocele cases and 2 cases with spina bifida and encephalocele. All UK families gave buccal swab samples collected using the protocol of Meulenbelt et al. [43] and completed questionnaires detailing the case's NTD type, maternal pregnancy history and family history of birth defects.

These cohorts are comparable in regards to folic acid supplementation. Of the Irish NTD mothers, 91 women definitively answered whether they were taking any folic acid or multivitamins in the month before their last monthly period; 23 (25.3%) were on supplements. Of the UK NTD mothers, 73 women definitively answered the same question; 23 (31.5%) were on supplements. Additionally, the distributions of case birth years in the two cohorts were similar and largely predated the recommendation of folate supplementation by the United States Public Health Service in 1992. Case birth years in the Irish NTD cohort ranged from 1938 to 2003 with a median of 1981 and a standard deviation of 11 years. Case birth years in the UK NTD cohort ranged from 1926 to 2001 with a median of 1978 and a standard deviation of 15 years.

The sample collections were approved by the Health Research Board Research Ethics Committee (Dublin, Ireland), the UK Multi-Centre Research Ethics Committee (MREC, University of Newcastle, UK) in collaboration with UK ASBAH, and the Institutional Review Board at the National Human Genome Research Institute (Bethesda, MD, USA). Written consent was obtained from all participants.

Genomic DNA was extracted from all blood samples and buccal swabs using the QIAamp DNA Blood Mini Kit kit (Qiagen).

African American control DNA (HD100AA) and Caucasian control DNA (HD200CAU) samples were purchased from the Coriell Cell Repositories.

SNP Selection and Discovery

To select a set of SNPs to capture common genetic variation in *TCblR*, we evaluated SNPs genotyped by HapMap [44]. SNPs within and up to 10kb from the gene were considered. A minimum SNP set representing all such HapMap SNPs was manually selected, while allowing exclusion of redundant SNPs (r^2>0.8).

Up to eighteen Irish individuals, the majority of which were drawn from NTD families, were screened for unreported polymorphisms. Primers were designed to PCR amplify exons 1 through 5; the resulting amplicons were analyzed via fluorescent automated dideoxy DNA sequencing.

Genotyping

Multiplex Ligation-dependent Probe Amplification (MLPA) was used to estimate the relative copy number for the 14.8kb copy number variant (CNV) immediately downstream from *TCblR*. Briefly, probe pairs were designed to hybridize to test and control loci (primer sequences available upon request). The multiplex reaction contains probe pairs to detect five test loci within the CNV. Control loci included four single copy regions in *TCblR* and one single copy probe was placed in *TCN2* for relative quantification. A locus in the androgen receptor (AR) was included as an additional control to ensure the method detects copy number differences of the X chromosome.

The MLPA reaction was performed as directed with SALSA kit reagents obtained from MRC-Holland (The Netherlands) using at least 50ng genomic DNA for each sample. After hybridization and ligation, products were PCR amplified and analyzed on an ABI 3100 Genetic Analyzer (Applied Biosystems). Comparisons of amplicon peak areas were used to estimate relative DNA quantity (DQ) scores for each locus. A real time PCR assay using the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems) was used to independently estimate copy number in a subset of samples with good correlation.

All other variants were genotyped by detection of allele-specific extension products via Matrix Assisted Laser Desorption/Ionization – Time of Flight (MALDI-TOF) mass spectrometry (Sequenom). Primer sequences and assay conditions are available upon request.

Genotyping quality was assessed in each sample population. More than 10% of Irish samples were repeated with ≥99% concordance for all variants. More than 8% of UK samples were repeated with ≥98% concordance for all variants. More than 7% of Caucasian (Coriell) samples were repeated with 100% concordance for all variants. More than 10% of African American (Coriell) samples were repeated with 100% concordance for all but two variants (E88del and rs9426, one discrepant sample each).

Genotyping call rates (i.e., success rates) averaged 97% for the Irish and UK samples. Call rates were at least 95% for all variants in all groups with the exception of rs4147651 in the Irish NTD cases (94%) and rs2232775 (R8Q) in the Irish controls (93%). Genotyping call rates averaged 98% in the Caucasian Coriell samples and 92% in the African American Coriell samples. Call rates were at least 93% for all variants in both Coriell groups with the exception of rs250503 (87%) in African Americans.

Only the NTD cases from Ireland were observed to deviate from Hardy-Weinberg equilibrium (HWE, $p<0.01$): rs2232775 (R8Q) NTD cases ($\chi^2=15.4$, $p=8.9\times10^{-05}$), rs2336573 (G220R) NTD cases ($\chi^2=27.8$, $p=1.3E^{-07}$), rs9426 NTD cases ($\chi^2=21.2$, $p=7.2E^{-06}$), and E88del NTD cases ($\chi^2=51.1$, $p=8.7E^{-13}$). These four SNPs were in HWE in all other genotyped groups. Additionally, the first three SNPs are in strong r^2 linkage disequilibrium ($r^2\geq0.94$), and similar genotyping results were obtained with these independent genotype assays. The E88del polymorphism has a low minor allele frequency ($p\sim0.02$), which may account for its failure to adhere to HWE in the NTD cases.

Discordant genotypes and triads exhibiting non-Mendelian inheritance for any single marker were excluded for that marker. Twenty-four samples with discrepancies for more than one marker were excluded from all analyses.

Haplotype analysis

Linkage disequilibrium (LD) in the region was estimated using Haploview (http://www.broad.mit.edu/mpg/haploview/, [45]. Haplotype blocks were defined based on D' values using the Solid Spine of LD option in Haploview. Haplotype frequency estimates based on these block definitions were then generated for Irish NTD cases, NTD mothers and controls using PHASE 2.1 [46, 47]. A permutation test within PHASE 2.1 was used to test whether haplotype frequency distributions differed between controls and NTD cases or NTD mothers in the Irish cohort.

Statistical Analyses

A logistic regression model, with the number of risk alleles as the independent variable, was used as a primary test to evaluate each polymorphism for NTD association. In this multiplicative model, the odds ratio of two risk alleles is the square of the odds ratio of one risk allele. Additionally, one degree of freedom (DOF) models of dominant and recessive genetic risk were applied via logistic regression. These tests are not valid when any of the genotypes cells are empty; in these cases, Fisher's Exact Test was used in place of the logistic regression to test for genotypic differences. These tests were used to perform NTD case-control comparisons as well as NTD mother-control comparisons in the Irish NTD cases, NTD mothers and controls.

The (mother, father, case) triads were analyzed by fitting multiplicative, dominant and recessive log-linear models with one DOF to test for case effects and one DOF to test for direct maternal effects [48]. The case term in the multiplicative log-linear model provides comparable information to the transmission-disequilibrium test (TDT) [49]. These analyses were modified to incorporate data from incomplete triads by using the expectation

maximization (EM) algorithm [50]. These family-based tests were applied to Irish and UK NTD triads to test *TCblR* variants for association with NTDs.

Correction for multiple tests was by permutation (99,999 random permutations). This method accounts for any linkage disequilibrium (non-independence) between evaluated SNPs. Multivariate permuting of triads for log-linear analysis involved treating the test as a one-sample test, and permuting the hypothetical risk allele. Permutations of cases and controls were independent of permutations of triads, and the results were combined by Bonferroni adjustment so that the resulting adjusted p-values accounted for all tests and all SNPs while controlling the chance of any false positive (familywise error) at 5%.

RESULTS

Genomic Analysis

The *TCblR* gene spans ~6kb on chromosome 19 (Figure 1A). It is flanked by a gene encoding a subunit of the mitochondrial NADH dehydrogenase, *NDUFA7*, and the LAG1 longevity assurance homolog 4 (*LASS4*) gene, which encodes a ceramide synthase. *TCblR* and *NDUFA7* are in close proximity (~3 kb) and transcribed from the same strand. In contrast, *LASS4* is transcribed from the opposite strand and its 3' terminus is mapped ~40kb away from the last exon of *TCblR*. The reference human genomic sequence appears contiguous through this region. Close examination of the genomic sequencing used to "build" this region of the genome revealed that the sequence in this interval had not been completed. The gap in the sequence was annotated in Genbank (contig, NT_077812) as including an undetermined number of a ~15kb repetitive element. During the course of this work, genome annotation was expanded to include areas containing potential segmental duplications. To ensure we were capturing all regional variability related to the *TCblR* gene, we first asked if this segmental duplication varied in copy number between individuals. Copy number variants (CNVs) have been implicated in disease and have been shown to affect expression for nearby genes. We developed a Multiplex Ligation-dependent Probe Amplification (MLPA) assay for this region and screened Caucasian and African American samples available from Coriell. We found that this region was polymorphic (i.e. this a bona fide CNV) in the African American (minor allele frequency, MAF=0.054) and Caucasian (MAF=0.021) samples tested. MLPA alone does not definitively allow determination of copy number; however, the most parsimonious model consistent with the MLPA derived data is that the common allele has two repeats while the minor allele has three repeats. The CNV was not found to be in strong LD with any other marker (Figure 1B). While it could serve as a candidate variant, we excluded it from further consideration because of its low MAF and the relatively large quantity of DNA consumed by the MLPA assay.

SNP analysis and Linkage Disequilibrium in the *TCblR* Region

We selected ten HapMap [44] polymorphisms to cover the region within and flanking *TCblR*. Additionally, while developing the MLPA assay we discovered a C>GA insertion-deletion (indel) polymorphism in the 3'UTR of exon 5 located 83 bases downstream from the stop codon. Lastly, to search for unreported common variation, limited screening of *TCblR* coding regions was performed by directly sequencing exons in at least twelve Irish

individuals. This revealed a three nucleotide insertion-deletion (indel) polymorphism in exon 2, resulting in the presence or absence of one of the three tandem glutamic acid (E) residues starting at codon 86 (*TCblR* E88del).

These twelve polymorphisms and the CNV were typed in Caucasians and African Americans to determine the linkage disequilibrium structure in the region (Figure 1B). Blocks defined by measures of D' revealed that the upstream region and the majority of *TCblR* are in a single block of LD (Figure 1). In the Irish population, this block encompassed all markers within *TCblR*. Most of the markers examined are independently informative ($r^2 < 0.8$). The exceptions consist of two sets of markers which are in high r^2 LD; 1) rs2232775 (R8Q), rs2336573 (G220R) and rs9426 ($r^2 \geq 0.94$); and 2) rs250511 and rs173665 ($r^2 = 0.99$). A total of twelve markers were genotyped in the Irish NTD cohort (Table 1). The exon 5 indel had the lowest MAF (0.001) and was excluded from further study.

Evaluation of Individual Markers for Association with NTDs

Each marker (n=11) was tested for case-control associations with NTDs in the Irish population using three models of logistic regression: a multiplicative model, a dominant model and a recessive model. Significant associations are shown in Table 2. Essentially, three positive associations were found. First, the three highly linked SNPs were found to be significantly associated in a recessive model of case effect (rs2232775 (R8Q), OR=10.6 [95% CI 1.28-88.56], p=0.029; rs2336573 (G220R), Fisher's Exact Test, p=0.0002; rs9426, Fisher's Exact Test, p=0.0006). These SNPs share very high r^2 values, and as predicted, yield similar association results. Second, *TCblR* E88del is also significantly associated with NTDs in a recessive model of case effect (OR=9.2 [95% CI 1.3-88.9], p=0.04). Third, the highly linked SNP pair (rs250511, OR=0.7 [95% CI 0.56-0.99], p=0.04; rs173665, OR=0.7 [95% CI 0.51-0.94], p=0.017) is significantly associated with NTDs in a dominant model of case effect. Additionally, rs173665 was associated when a multiplicative model of case effect was applied (OR=0.7 [95% CI 0.55-0.96], p=0.02). Upon correcting for multiple tests, two markers from the highly linked trio remained significant: rs2336573 (G220R) (p=0.008) and rs9426 (p=0.03), by Fisher's Exact Test of a recessive model in cases vs. controls. No other comparisons were significant after adjusting for multiple tests.

Examining the same markers in NTD mothers and controls revealed no significant associations (data not shown).

A family-based test of association was also used to evaluate these eleven markers for case and maternal NTD risk (Table 3). Multiplicative (data not shown), dominant and recessive models of log-linear analysis were applied. Depending on the applied model and corresponding genotype frequencies, the test failed to converge (no result obtained) for some SNPs. One positive result was observed among the successfully tested SNPs. All three highly linked SNPs were significantly associated with a strong relative risk in log-linear analysis of the recessive model in cases (rs2232775 (R8Q), RR=6.0, p=0.0086; rs2336573 (G220R), RR=6.6, p=0.0037; and rs9426, RR=6.7, p=0.0035). However, these results did not withstand correction for multiple tests (data not shown).

Further evidence was sought by evaluating *TCblR* variation for NTD risk in an independent population. Seven SNPs were tested in a United Kingdom cohort of NTD triads (Table 3). Again, some tests failed to converge, including log-linear analysis of the recessive model as applied to two of the associated SNPs (rs2232775 (R8Q) and rs2336573 (G220R)). The remaining models of SNPs that were successfully tested were not found to be associated with case or maternal NTD risk in the UK population.

Haplotype Analysis of *TCblR* Variants for NTD Risk

To test for *TCblR* haplotype association, a haplotype was constructed based on the single block of LD in the Irish population (Figure 1B). rs2336573 (G220R) was retained to tag rs2232775 (R8Q) and rs9426, which were not included in these analyses. Similarly, rs173665 was retained as a tag for rs250511. The resulting haplotype block is defined by the remaining seven markers: rs4147651, rs7408841, rs2927707, E88del, rs2336573 (G220R), rs2227288, rs173665. Haplotype frequencies were estimated in Irish controls, NTD cases and mothers (Table 4). Five haplotypes account for >90% of the variation present in this haplotype block in the Irish population. A permutation test did not detect differences in haplotype frequencies between NTD cases and controls (p=0.3) or NTD mothers and controls (p=1.0).

DISCUSSION

We evaluated the association between genetic variation in the transcobalamin II receptor gene, *TCblR*, and NTD risk. When considered singly, several independent (r^2<0.8) *TCblR* polymorphisms were implicated as NTD risk factors in the Irish population. One pair of redundant SNPs (rs2336573 (G220R) and rs9426, r^2~1) remained significantly associated with NTDs even after rigorous correction for multiple tests. One possible concern about the validity of this result is that the NTD cases are not in HWE for these SNPs (see Methods), which can result from genotyping error. We consider this unlikely because these two highly linked SNPs were genotyped with two independent assays, producing concordant results. Moreover, these variants were also significantly associated in an unadjusted recessive model of log-linear analysis that does not use control data, eliminating the possible problem of population stratification. Furthermore, this finding may not have withstood correction because power is reduced when examining a variant with a low MAF. Thus, we conclude that the signal seen with the rs2336573 (G220R) and rs9426 variants represents a true genetic risk factor in *TCblR* in the Irish population.

We sought to replicate this association in a sample of triads from the UK. As this sample did not include population controls, we were restricted to family-based tests of association. The lack of individuals homozygous for the minor allele of rs2336573 (G220R) precluded the use of the log-linear test for a recessive case effect. Because testing of the Irish cohort revealed a case effect, we also performed the TDT [49] but did not detect a case effect for either rs2336573 G220R or rs2232775 R8Q in the UK cohort (data not shown). The rs9426 SNP was not directly tested, but due to its high LD with the other two markers we would predict the same result in the UK NTD cohort. This lack of replication may be due to decreased power to detect an effect (~525 Irish NTD triads vs. ~345 UK NTD triads).

Alternatively, because of differences in dietary factors, prenatal screening and/or greater ethnic heterogeneity, these variants may not contribute to NTDs in the UK population.

Haplotype analysis of all *TCblR* variants did not yield further evidence of association; therefore the identified SNP pair exhibits the strongest signal for NTD risk. It is possible that these variants do not alter gene function and a yet unidentified "causal" risk SNP may be linked to these two and reside on a haplotype we have yet to test. In addition, these SNPs (rs2336573 G220R and rs9426) have been typed in the HapMap CEU (Caucasian) population, and are in very high LD (r^2~1) with five additional *TCblR* SNPs: rs17160390 (intron 1), rs2232783 (T149T), rs2232784 (S161S), rs2232785 (intron 3) and rs2227289 (T279T). Additionally, there are four such SNPs in introns of upstream genes: rs7249111, rs7250792, rs2288414 in *NDUFA7* and rs4147645 in ribosomal protein S28 (*RPS28*).

We aligned the *TCblR* protein sequences from multiple species to determine whether any of these variants are in highly conserved regions. The "Conservation" track of the University of California - Santa Cruz Genome Browser (http://genome.ucsc.edu/; [51] displays syntenic regions of up to 17 species, and full alignments for *TCblR* were obtained only for chimp, rhesus, dog, cow, mouse, rat, tenrec and elephant. The only intronic SNP with a non-zero phastCons conservation score is rs17160390 (*TCblR* intron 1); its signal is relatively low. In contrast, rs2232783 (T149T), rs2232784 (S161S) are the only other SNPs in this set with phastCons conservation scores (0.742 and 0.677, respectively). These SNPs are part of the coding region for the second Low Density Lipoprotein Receptor Class A (LDLRa) domain in *TCblR*. As the name implies, these domains are shared by proteins in the LDL receptor superfamily, which includes the LDL receptor (*LDLR*), the LDL receptor related protein (*LRP1*), megalin/*LRP2*, the apolipoprotein 2 receptor (*LRP8*), and the VLDL receptor (reviewed in [52]). The LDLRa domains shared by these proteins contain cysteine-rich regions of approximately 40 amino acids and are involved in ligand binding. Whilst rs2232783 (T149T) and rs2232784 (S161S) fall into this well-conserved region, they are synonymous coding SNPs and appear to be weak candidates for affecting functionality.

Perhaps the most likely causal candidate SNP is rs2336573 (G220R), the nonsynonymous coding SNP directly tested in this study. This amino acid substitution replaces a glycine with arginine and is predicted to reside ten residues from the transmembrane and cytoplasmic domains found at the carboxyl terminus of the protein. We aligned and examined the *TCblR* orthologs from 23 mammalian species. This position contains glycine in 14 species. The other species contain glutamic acid, arginine, alanine or tryptophan at this residue. The moderate conservation of this amino acid implies functional tolerance of the rs2336573 (G220R) variant in *TCblR* While such speculation is intriguing, functional analyses of these individual variants will be required to determine which of these NTD-associated SNPs may be the direct contributor to NTD risk.

Strengths of the current study include a large, homogenous Irish population, as well as a large UK population for replication. One limitation was the lack of UK population-based controls, which prevented replication of the same case-control analyses that identified rs2336573 (G220R) and rs9426 as NTD risk SNPs in the Irish group. Thus replication studies in independent cohorts are required to determine whether these *TCblR*

polymorphisms contribute to NTDs in other populations. Additionally, the lack of metabolite measurements in either population prevented testing whether the risk genotype influences circulating cobalamin levels.

In summary, we identified a highly linked SNP pair (rs2336573 (G220R) and rs9426) in *TCblR* that is significantly associated in a recessive model of NTD case risk. The risk genotype (minor allele homozygote) was undetected in the large control group (n=979), contributing to the large relative risk (RR~6) observed in NTD cases. As a complex disease, multiple genetic risk factors are expected to contribute to the development of NTDs. Because of the relative rarity of the TCblR 2336573 (G220R) and TCblR rs9426 risk genotypes (~1% in NTD cases), we lack the power to perform meaningful interaction analyses of these genotypes with other established ntd risk factors such as MTHFR 677C>T. Its low frequency also means the risk genotype does not contribute greatly to the total genetic risk for NTDs in the general population. However, for those few individuals carrying the risk genotype, risk is increased ~6-fold.

Finding a genetic risk factor in the receptor required for cobalamin bioavailability further establishes the role of cobalamin in the development of NTDs. This raises the possibility that women with the risk variant may need additional dietary cobalamin to provide sufficient intracellular cobalamin for the developing embryo at the time of neural tube closure. There may also be individuals with these polymorphisms in the general population who need dietary supplements to maintain normal cobalamin homeostasis. These identified NTD risk SNPs are prime candidates to investigate in any disease state influenced by cobalamin metabolism.

Acknowledgments

These studies would not be possible without the participation of the affected families, and their recruitment by the Irish Association of Spina Bifida and Hydrocephalus and the Irish Public Health Nurses in Ireland, and by the UK Association for Spina Bifida and Hydrocephalus. The authors would like to thank Amanda Samuels and David Bernard for technical assistance in screening *TCblR* coding regions for polymorphisms.

Funding: The authors acknowledge research support from the intramural research programs of the National Human Genome Research Institute, the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, NIH, DHHS and the Health Research Board, Ireland. EVQ and JMS are supported by NIH grant DK064732.

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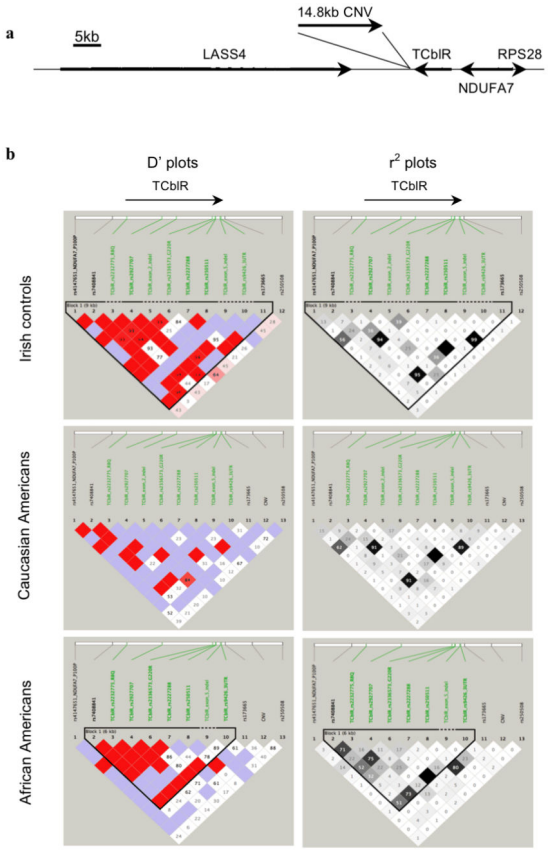

Figure 1.

Genomic Structure and Linkage Disequilibrium in the *TCblR* Region. A) Genomic structure of the region including *TCblR*. Orientation, relative size and distance of *LASS4* (53kb), the 14.8kb CNV, *TCblR* (6kb), *NDUFA7* (10kb) and *RPS28* (0.9kb) are shown. The 14.8kb CNV is shown in single copy, although two copies are predicted to be present on most chromosomes. B) Linkage disequilibrium plots of polymorphisms within and surrounding *TCblR*. Genotyping data from Irish controls (n=993), Caucasian Americans (n=79) and African Americans (n=83) were used to construct D' and r^2 LD plots. Labels for variants within *TCblR* are green. Relative distances between markers are shown with two exceptions: the CNV is approximately 450 bases downstream from *TCblR*, and rs250508 is approximately 32kb downstream from *TCblR*.

Table 1

Genotype Distributions and Allele Frequencies in Irish Controls and NTD Triads*

Marker	Controls	NTD Children	NTD Mothers	NTD Fathers
Genotypes	N (freq.)	N (freq.)	N (freq.)	N (freq.)
Alleles	Freq.	Freq.	Freq.	Freq.
NDVFA7				
rs4147651				
GG	636 (0.66)	314 (0.63)	310 (0.62)	289 (0.64)
GA	290 (0.30)	167 (0.33)	175 (0.35)	140 (0.31)
AA	31 (0.03)	21 (0.04)	16 (0.03)	24 (0.05)
G	0.82	0.79	0.79	0.79
A	0.18	0.21	0.21	0.21
rs7408841				
GG	377 (0.39)	210 (0.41)	223 (0.44)	193 (0.42)
GC	459 (0.48)	218 (0.43)	218 (0.43)	201 (0.44)
CC	129 (0.13)	81 (0.16)	63 (0.13)	61 (0.13)
G	0.63	0.63	0.66	0.65
C	0.37	0.37	0.34	0.35
TCblR				
rs2232775				
R8Q				
TT	846 (0.91)	479 (0.91)	479 (0.93)	423 (0.91)
TC	82 (0.09)	44 (0.08)	33 (0.06)	39 (0.08)
CC	0 (0.00)	6 (0.01)	2 (0.00)	3 (0.01)
T	0.95	0.95	0.96	0.95
C	0.05	0.05	0.04	0.05
TCblR				
rs2927707				
TT	481 (0.50)	259 (0.51)	243 (0.48)	227 (0.50)
TC	409 (0.43)	212 (0.41)	212 (0.42)	189 (0.42)
CC	71 (0.07)	41 (0.08)	51 (0.10)	38 (0.08)
T	0.71	0.71	0.69	0.71
C	0.29	0.29	0.31	0.29
TCblR				
E88del				
E/E	915 (0.96)	495 (0.95)	493 (0.97)	436 (0.96)
E/del	35 (0.04)	20 (0.04)	15 (0.03)	18 (0.04)
del/del	0 (0.00)	5 (0.01)	0 (0.00)	0 (0.00)
E	0.98	0.97	0.99	0.98
del	0.02	0.03	0.01	0.02
TCblR				
rs2336573				

Marker	Controls	NTD Children	NTD Mothers	NTD Fathers
G220R				
CC	889 (0.92)	464 (0.90)	468 (0.93)	417 (0.91)
CT	80 (0.08)	42 (0.08)	35 (0.07)	39 (0.08)
TT	0 (0.00)	8 (0.02)	1 (0.00)	3 (0.01)
C	0.96	0.94	0.96	0.95
T	0.04	0.06	0.04	0.05
TCblR				
rs2227288				
GG	780 (0.81)	417 (0.82)	404 (0.80)	364 (0.80)
GC	179 (0.19)	90 (0.18)	97 (0.19)	89 (0.20)
CC	6 (0.01)	4 (0.01)	6 (0.01)	2 (0.00)
G	0.90	0.91	0.89	0.90
C	0.10	0.10	0.11	0.10
TCblR				
rs250511				
TT	802 (0.82)	453 (0.86)	428 (0.83)	392 (0.84)
TA	175 (0.18)	72 (0.14)	83 (0.16)	76 (0.16)
AA	7 (0.01)	4 (0.01)	6 (0.01)	0 (0.00)
T	0.90	0.92	0.91	0.84
A	0.10	0.08	0.09	0.16
***TCblR* exon 5**				
indel				
del/del	982 (1.00)	508 (1.00)	504 (1.00)	456 (1.00)
del/ins	1 (0.00)	1 (0.00)	1 (0.00)	1 (0.00)
ins/ins	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
del	1.00	1.00	1.00	1.00
ins	0.00	0.00	0.00	0.00
***TCblR* rs9426**				
CC	882 (0.91)	470 (0.90)	473 (0.93)	425 (0.91)
CT	87 (0.09)	44 (0.08)	35 (0.07)	39 (0.08)
TT	0 (0.00)	7 (0.01)	1 (0.00)	3 (0.01)
C	0.95	0.94	0.96	0.95
T	0.05	0.06	0.04	0.05
rsl73665				
GG	790 (0.82)	445 (0.86)	421 (0.83)	389 (0.84)
GA	171 (0.18)	66 (0.13)	79 (0.16)	74 (0.16)
AA	8 (0.01)	4 (0.01)	6 (0.01)	1 (0.00)
G	0.90	0.93	0.91	0.92
A	0.10	0.07	0.09	0.08
rs250508				
CC	304 (0.32)	176 (0.35)	166 (0.34)	140 (0.30)
CT	482 (0.50)	222 (0.44)	235 (0.48)	222 (0.47)

Marker	Controls	NTD Children	NTD Mothers	NTD Fathers
TT	169 (0.18)	107 (0.21)	87 (0.18)	107 (0.23)
C	0.57	0.57	0.58	0.54
T	0.43	0.43	0.42	0.46

* Due to rounding, group frequencies may not sum to 1.

Table 2

Case-Control Logistic Regression of Significantly Associated *TCblR* Variants in the Irish Population.

		Logistic regression			Fisher's Exact Test	
	Model	**OR* (95% CI)**	**p-value**	**corrected p-value**	**p-value**	**corrected p-value**
rs2232775 (R8Q)	Recessive	10.6 (1.28-88.56)	0.0288	0.3747		
E88del	Recessive	9.2 (1.08-79.16)	0.0428	0.6887		
rs2336573 (G220R)	Recessive	FTC**			0.0002	0.0080
rs250511	Dominant	0.7 (0.55-0.99)	0.0422	1.0000		
rs9426	Recessive	FTC			0.0006	0.0279
rsl73665	Dominant	0.7 (0.51-0.94)	0.0170	0.7217		
rsl73665	Multiphcative	0.7 (0.55-0.96)	0.0249	1.0000		

* OR for the Recessive Model is the odds of disease with 2 copies divided by odds of disease with 0 or 1 copy; OR for the Dominant Model is the odds of disease with 1 or 2 copies divided by odds of disease with 0 copies; OR for the Multiplicative Model is the odds ratio for disease for each copy of the allele.

** FTC = failed to converge

Table 3

Dominant and Recessive Models of Log-Linear Analysis *of TCblR* Variants

	Republic of Ireland				United Kingdom			
Variant	**Dom RR***	**Dom p-value**	**Rec RR**	**Rec p-value**	**Dom RR**	**Dom p-value**	**Rec RR**	**Rec p-value**
rs4147651								
Case	1.1048	0.4329	0.7598	0.3496	FTC**	FTC	0.6830	0.3386
Mother	1.0342	0.5795	0.5868	0.1055	0.9765	0.5303	1.0637	0.8976
rs7408841								
Case	FTC	FTC	1.3588	0.0824	1.2350	0.2127	1.0259	0.9091
Mother	FTC	FTC	0.8482	0.4135	1.1464	0.3378	0.8033	0.3594
rs2232775								
Case	FTC	FTC	6.000	0.0086	0.7965	0.4081	FTC	FTC
Mother	FTC	FTC	0.4567	0.3357	0.8394	0.1738	FTC	FTC
rs2927707								
Case	1.0561	0.6148	0.7133	0.1091	0.9483	0.7146	0.9659	0.9014
Mother	FTC	FTC	1.2461	0.3263	0.9582	0.6013	0.8331	0.4988
E88del								
Case	1.3405	0.7462	FTC	FTC	ND***	ND	ND	ND
Mother	FTC	FTC	FTC	FTC	ND	ND	ND	ND
rs2336573								
Case	FTC	FTC	6.5871	0.0037	0.7064	0.2550	FTC	FTC
Mother	FTC	FTC	0.2469	0.1858	0.7518	0.1386	FTC	FTC
rs2227288								
Case	1.0210	0.8971	0.6856	0.4932	0.8320	0.3962	0.4258	0.2315
Mother	FTC	FTC	2.6990	0.2006	0.8055	0.5434	0.4428	0.3369
rs250511								
Case	0.7980	0.2625	FTC	FTC	ND	ND	ND	ND
Mother	FTC	FTC	FTC	FTC	ND	ND	ND	ND
rs9426								
Case	FTC	FTC	6.7127	0.0035	ND	ND	ND	ND
Mother	FTC	FTC	0.2471	0.1656	ND	ND	ND	ND
rsl73665								
Case	0.7253	0.1313	FTC	FTC	0.9480	0.8021	0.7332	0.7084
Mother	FTC	FTC	FTC	FTC	FTC	FTC	2.4150	0.4509

	Republic of Ireland				United Kingdom			
Variant	**Dom RR***	**Dom p-value**	**Rec RR**	**Rec p-value**	**Dom RR**	**Dom p-value**	**Rec RR**	**Rec p-value**
rs250508								
Case	0.8428	0.1861	1.2416	0.1621	ND	ND	ND	ND
Mother	0.9832	0.6401	0.7748	0.1239	ND	ND	ND	ND

Note: none of the reported p-values are <0.05 upon correction for multiple tests (permutation)

* RR for the Recessive Model is the risk of disease with 2 copies divided by risk of disease with 0 or 1 copy; RR for the Dominant Model is the risk of disease with 1 or 2 copies divided by risk of disease with 0 copies.

** FTC = Failure to converge

*** ND = not done

Table 4

Haplotype Frequency Estimates of the *TCblR* D' Block*

Haplotype	Controls	NTD cases	NTD mothers
GCTdelCGG	0.33	0.32	0.30
GGTdelCGG	0.24	0.24	0.24
AGCdelCGG	0.19	0.21	0.21
GGTdelCCG	0.10	0.10	0.11
GGCdelCGA	0.10	0.07	0.09
GCTdelTGG	0.02	0.03	0.02
GCTinsTGG	0.02	0.03	0.01
GGCdelCGG	0.01	0.01	0.01
p-value		0.3**	1.0**

*The haplotype block consists of the following markers: rs4147651, rs7408841, rs2927707, E88del, rs2336573 (G220R), rs2227288, rsl73665.

**Result of permutation test for significant differences in haplotype frequencies in NTD cases vs. controls or NTD mothers vs. controls.