Published in final edited form as:

Am J Med Genet A. 2009 February ; 149A(2): 155–160. doi:10.1002/ajmg.a.32589.

Genetic Polymorphisms in the Thioredoxin 2 (*TXN2*) Gene and Risk for Spina Bifida

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Abstract

TXN2 encodes human thioredoxin 2, a small redox protein important in cellular antioxidant defenses, as well as in the regulation of apoptosis. *Txn2* knockout mice fail to complete neural tube closure by E10.5 and die *in utero*. We hypothesized that genetic variation in human *TXN2* gene may alter the function of the encoded protein in a manner associated with an increased risk for neural tube defects (NTDs). A DNA re-sequencing effort of the human *TXN2* gene was taken. After a variation in the promoter was identified, the transcriptional activity of different alleles was investigated. The possible association between these variations and the risk of spina bifida was further evaluated in a subset of samples obtained from a large population-based case-control study in California in two different ethnicity groups, non-Hispanic white and Hispanic white. We identified a novel promoter insertion polymorphism located 9 base pairs upstream of the transcription start site of exon 1 (–9 insertion). The GA, G and GGGA insertions were associated with a marked decrease of transcriptional activity when overexpressed in both U2-OS (an osteosarcoma cell line) and 293 cells (derived from human embryonic kidney). Further analysis revealed that the GA insertion was associated with increased spina bifida risk for Hispanic whites. Our study revealed a novel Ins/Del polymorphism in the human *TXN2* gene proximal promoter region that altered the transcriptional activity and is associated with spina bifida risk. This polymorphism may be a genetic modifier of spina bifida risk in this California population.

Keywords

thioredoxin 2; *TXN2*; re-sequencing; Ins/Del polymorphism; spina bifida

INTRODUCTION

Neural tube defects (NTDs) are a group of severe congenital malformations characterized by a failure of neural tube closure during early embryonic development [Blom et al., 2006]. Both genetic and environmental factors are believed to contribute to the etiology of NTDs [Detrait et al., 2005]. Periconceptional folic acid supplementation is believed to be highly beneficial when provided in the periconceptional period and has been credited with the

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ability to prevent 50–70% of all NTDs [Berry et al., 1999; Czeizel and Dudas, 1992; MRC, 1991]. Mutations and polymorphisms in folate pathway genes such as 5,10-methylenetetrahydrofolate reductase (*MTHFR*), methionine synthase (*MTR*), and methionine synthase reductase (*MTRR*) have been intensely studied, and in some cases, associated with elevated risk of NTDs [Barber et al., 2000; Botto and Yang, 2000; Shaw et al., 1998; Zhu et al., 2003]. However, none of the known folate pathway gene variants contributes substantially to the population burden of NTDs [Kibar et al., 2007]. Thus, efforts continue to identify genes that contribute a substantial portion of the population risk for spina bifida.

There have been several other developmental mechanisms postulated to contribute to abnormal development of the neural tube. Animal models have provided crucial mechanistic information at the cellular and tissue levels with regards to how embryos close their neural tubes [Harris and Juriloff, 1999]. More than 150 genetic mouse models exhibit NTDs, with new ones emerging from gene targeting studies and large scale mutagenesis screens on a regular basis [Blom et al., 2006]. A survey of the genes whose disruption causes NTDs suggests multiple key signaling pathways and cellular functions that are essential for neural tube closure. One such candidate involves mitochondrial thioredoxin 2, a small reactive oxidative stress protein that appears to play an important role in normal mouse embryonic development. Thioredoxin 2, encoded by the *TXN2* gene (*Txn2* in mice), contains the active site Trp-Cys-Gly-Pro-Cys-Lys; the cysteine residues function to maintain protein thiols in a reduced state, and thereby contribute to the mitochondria's antioxidant defenses. In addition to protecting the cell against damage from reactive oxygen species (ROS), *TXN2* also plays an important role in regulating cellular apoptosis. For example, *TXN2* protects against oxidative damage triggered by TNF-alpha in HeLa cell by blocking TNF-alpha-induced ROS generation and apoptosis [Hansen et al., 2006]. Abnormal function of *TXN* system has been associated with a variety of pathological conditions, such as cataract formation, ischemic heart diseases, cancers, AIDS, complications of diabetes, etc. [Maulik and Das, 2008].

Inactivation of the *Txn2* gene in mice results in failure of neural tube closure E10.5. The homozygous mutant embryos display an open anterior neural tube and show massively increased apoptosis at 10.5 days post-conception and are not present by 12.5 days post-conception [Nonn et al., 2003]. There is also a wealth of literature suggesting that mitochondrial damage resulting from overproduction of ROS can lead to the development of a variety of degenerative diseases [Martin, 2006]. Phenotypic studies of mouse embryos in which the *Txn2* gene had been inactivated demonstrated a failure of anterior neural tube closure. Furthermore, Western Blot analysis confirmed the lack of *Txn2* protein in the homozygous mutant embryos. These findings suggest that variation in the *Txn2* gene alters protein function in a manner associated with an increased risk for NTDs.

The human *TXN2* gene (NT_011520), which maps to chromosome 22, contains four exons and encodes an 18 kDa protein composed of 166 amino acids. Human *TXN2* gene shares 82.44% homology with its mouse ortholog. In this study, we re-sequenced the exons and proximal promoter region of the human *TXN2* gene, and tested the hypothesis that genetic polymorphisms in *TXN2* may modify human spina bifida risk. This hypothesis was evaluated in a population-based case-control study of infants with spina bifida and non-malformed controls.

MATERIALS AND METHODS

Subjects

Study participants were provided in collaboration with the California Birth Defects Monitoring Program, a population-based active surveillance system for collecting

information on infants and fetuses with congenital malformations [Croen et al., 1991]. Program staff collected diagnostic and demographic information from multiple sources of medical records for all live-born or stillborn (defined as >20 weeks gestation) fetuses, and pregnancies electively or spontaneously terminated. Nearly all structural anomalies diagnosed within one year of delivery were ascertained. Overall ascertainment has been estimated as 97% complete [Schulman et al., 1993].

Included for study were 48 infants with spina bifida (cases) and 48 non-malformed infants (controls). Among the 48 controls, 30 (62.5%) were non-Hispanic white, 10 (20.8%) were Hispanic white, and 8 (16.7%) were of other ethnicities (African American, Asian, etc.). Among the 48 cases, 24 (50%) were non-Hispanic white, 17 (35.4%) were Hispanic white, and 7 (14.6%) were of other ethnicity (African American, Asian, etc.). These cases and controls were derived from 1983–86 birth cohorts in selected California counties. Each case and control infant was linked to its newborn bloodspot, which served as the source of DNA in our genotyping analysis. All samples were obtained with approval from the State of California Health and Welfare Agency Committee for the Protection of Human Subjects. Genomic DNA was extracted from dried newborn screening bloodspots using the Puregene DNA Extraction Kit (Gentra, Minneapolis, MN) and quantitated by TaqMan RNase P Control Reagents (AppliedBiosystems, Foster City, CA).

Sequence Analysis of TXN2 gene—Exons and the proximal promoter region of the *TXN2* gene were re-sequenced in 48 cases and 48 controls to identify novel sequence variants of the target genome region that were not present in existing databases. Primers covering the four exons and proximal promoter region were designed based on region 16129455–16229445 (GenBank accession number NT_011520), using the online program Primer3 (Whitehead Institute for Biomedical Research, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) [Rozen and Skaletsky, 2000] (Table I). PCRs were performed at preferred annealing temperature in a final volume of 25 μ l containing 60ng genomic DNA, 2.0 μ l primer mix, 250 μ M of each dNTP, in 2.0mM MgCl₂, 50mM KCl, 20mM Tris-HCl (pH 8.4), and 1.5 U of Taq DNA polymerase in a PE9700 thermal cycler (AppliedBiosystems, Foster City, CA). The PCR products were cleaned up by digestion with ExoSAP-IT (USB Corporation, Cleveland, Ohio), and applied to the sequencing reaction (10 s at 96°C, 5 s at 50°C, 4 m at 60°C for 25 cycles) with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The fragments were precipitated with isopropanol, denatured with HiDi Formamide, and loaded onto an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). Sequencing results were exported to Sequencher™ software version 4.2.2 (Gene Code Corp., Ann Arbor, MI) for alignment and multiple comparisons. After the polymorphic region was identified, each allele was subsequently sub-cloned into a TA vector (Invitrogen, San Diego, CA) and sequenced from both sides.

Statistical Analysis—Spina bifida risks were measured by both genotypic and allelic association, using odds ratios (ORs), which are useful in measuring the size of an effect in case-control studies. Risk estimation was computed by logistic regression models utilizing SAS software (version 9.1). The models were adjusted for race/ethnicity (defined as non-Hispanic white, Hispanic white, and other).

Promoter analysis—The sequences of the polymorphism flanking region were analyzed with P-Match and TRANSFAC databases [Chekmenev et al., 2005] for potential transcriptional factor binding sites.

In vitro transient transfection—Transient transfection studies were performed in U2-OS and 293 cells using the TransFast Reagent (Promega, Madison, WI). U2-OS cell is an

osteosarcoma cell line that could be used as an in vitro model system for bone formation during development [Wallin et al., 1990]. The 293 cell line is derived from a human embryonic kidney that is widely used as a tool for exogenous gene expression [Graham et al., 1977]. Both osteosarcoma cells and 293 cells are known for endogenous *TXN2* expression and protection against oxidative stress and apoptosis [Chen et al., 2002; Damdimopoulos et al., 2002]. Thus, these two cell lines appeared to be appropriate tools with which to study the transcription regulation of the *TXN2* gene. Triple tandem repeats of the polymorphism region were constructed with Oligonucleotides (IDTDNA, Coralville, Iowa) as listed in Table II. Each pair of complementary oligonucleotides were mixed and denatured at 95°C for 5 minutes, then annealed gradually to room temperature, and subcloned into a firefly luciferase reporter vector, pGL3-promoter (Promega, Madison, WI). The insertions were confirmed by automated DNA sequencing analysis. The renilla luciferase vector (Promega, Madison, WI) was co-transfected with target constructs. Successful transfection was demonstrated by firefly luciferase activity as read by a FLUOstar OPTIMA (BMG Lab Technologies, Durham, NC). The in vitro transcription activity was normalized by renilla luciferase activity. Each experiment was repeated a minimum of three times.

RESULTS AND DISCUSSION

The promoter and exons of the *TXN2* gene in the 48 cases and controls were sequenced. The insertion/deletion polymorphism observed in the human *TXN2* gene was located in the proximal promoter region, 9 base pairs upstream of the transcription start site of exon1. This polymorphism has been reported previously as an unvalidated A insertion (rs35045487). Five alleles were identified through our DNA re-sequencing effort: no insertion (reference) (Allele 1, or A1), GA insertion (Allele 2, or A2), AA insertion (Allele 3, or A3), G insertion (Allele 4, or A4), and GGGA insertion (Allele 5, or A5) (Figure 1). The novel polymorphism was submitted to NCBI as *TXN2_intron2_GAins*, rs35045487.

We subsequently tested the activity of the various promoter alleles by transient transfection of promoter allele–luciferase constructs into U2-OS and 293 cells. After transient transfection, we observed decreases in transcriptional activity for the A2, A4 and A5 alleles when compared to the A1 allele in both U2-OS and 293 cells. When analyzing the polymorphism flanking region with P-Match and Transfac [Chekmenev et al., 2005], A2, A4, and A5 alleles all exhibited one or more extra SP1 binding sites, compared to the A1 or A3 alleles. It is known that transcription factors SP1 and Myc-associated zinc finger protein (MAZ) could both bind to the SP1 binding site and act as repressors for gene expression [Song et al., 2001]. Thus, these extra SP1 sites might be responsible for the observed decreased transcriptional activity for the A2, A4, and A5 alleles.

We evaluated the association between the *TXN2* gene and spina bifida risk in the two major ethnic subpopulations: non-Hispanic and Hispanic whites. The results of the two common alleles (A1 and A2) are presented in Table III. A3, A4, and A5 present the rare allelic forms of the *TXN2* gene. One case infant was found to be homozygous for the A4 allele, another case infant was heterozygous for the A5 genotype. The A5 allele was not found among controls. Among Hispanic whites, the A2 allele was associated with an increased, albeit imprecise, risk for spina bifida (OR=1.5, 95%CI=0.5–5.0).

We discovered and characterized activity changes associated with this novel ins/del polymorphism in the proximal promoter region of the human *TXN2* gene. To our knowledge, this is also the first report evaluating a possible association between the human *TXN2* gene polymorphism and spina bifida risk. Previous studies have investigated the association between three intronic SNPs in *TXN2* gene and risk or prognosis of breast

cancer, but they failed to detect any association [Cebrian et al., 2006; Oestergaard et al., 2006; Udler et al., 2007]. Yet these SNPs are not in linkage disequilibrium with the polymorphism we identified and may not provide any information about this polymorphism. Our study provided a direct correlation between the distribution of these alleles in a patient population and a functional biochemical consequence of these alleles, in vitro. Infants carrying the A2 allele had an increased, although very imprecise due to limited sample size, spina bifida risk in the overall population, particularly among Hispanic whites. The observed increases in spina bifida risk indicated a possible role of mitochondrial maturation in human neural tube morphogenesis.

One possible pathogenetic mechanism underlying the observed results may be that the altered *TXN2* gene may result in faulty regulation of apoptosis [Husemoen et al., 2006]. During early embryonic development, the neuroepithelium must develop properly for the neural folds to meet and fuse appropriately [Chi et al., 2005]. It has been shown in multiple mouse models that in the presence of excessive cell death, the anterior [Tang and Finnell, 2003; Tang et al., 2004] and posterior [Wong et al., 2008] neural tube remain open. Studies have shown that the *TXN2* gene is critical in regulating mitochondria-dependent apoptosis [Lwin et al., 2002], and that such apoptosis is caused by an overproduction of reactive oxygen species (ROS) within the mitochondria [Nonn et al., 2003]. The *TXN2* gene is a nuclear gene that encodes the thioredoxin-2 system and plays an important role in the mitochondria's antioxidant defenses. The protein is especially abundant in actively respiring cells of the brain, heart and liver. When the protein is deficient, ROS buildup in the mitochondria leads to oxidative damage to biological molecules and excessive apoptosis, which has previously been implicated in abnormal neural tube closure [Nonn et al., 2003]. Our study, from both the population and experimental perspectives, suggested that genetic polymorphism of *TXN2* in early embryos may contribute to the risk of human spina bifida.

The strengths of this study include its population-based ascertainment of cases and controls and its evaluation of race/ethnicity as potentially important modifiers of risk in the presence of variant polymorphisms. Allele association analysis suggested that the A2 allele, which contained a GA insertion and lowered the promoter activity, conferred an increased risk of spina bifida, especially in Hispanic whites. A4 and A5 also had lower promoter activity, and they were the least prevalent alleles in the study population. There was one A4 homozygote and one A5 heterozygote found among the spina bifida infants, while no control infants were found to have these genotypes. Decreased transcriptional activity for A2 alleles provided additional molecular evidence to support our hypothesis that *TXN2* is associated with spina bifida risk.

Our results, although based on small sample sizes and therefore of limited statistical power, represent a preliminary step in elucidating the association between *TXN2* gene variations and spina bifida risk. Combined with existing knowledge about the etiology of NTDs, a larger, more robust study of the *TXN2* promoter polymorphism could contribute to understanding the complex mechanism underlying genetic susceptibility for NTDs. Further studies of these variations using animal models and cellular experiments could potentially provide an in-depth view of the mechanism suggested by our genetic observations.

Acknowledgments

The authors appreciate the technical support of Ms. Consuelo Vega and Ms. Dia Gentile. This work was supported in part by funds from NIH grant NS050249-01, and support from the Centers for Disease Control and Prevention U59/CCU913241. Additional support was provided by the Margaret M. Alkek Foundation.

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Figure 1. Sequence chromatograph. A1:reference sequence; A2:GA insertion; A3:AA insertion; A4:G insertion; A5: GGGG insertion. Red dots indicate location of polymorphism.

Figure 2.

Distribution of promoter activity of transfected *TXN2* promoter alleles in U2-OS and 293 cells. Activity is presented as the ratio of firefly luciferase and renilla luciferase activities relative to A1 (set to 1), including standard deviation. Repeated transfections were carried out in at least triplicate with different batches of cells. A similar activity pattern of alleles was observed in both U2-OS and 293 cells.

Table I

Primer sequences for re-sequencing of TXN2 gene

Region	Primer sequence	Annealing temperature	Product size (bp)
Exon 1	Fw: 5'-GACCCTGGCTCTCCGGCGAG Re: 5'-GAGGGACGCTCTCCGCCTG	65	255
Exon 2	Fw: 5'-GCAGCATAGAACATGGCCGTG Re: 5'-CTGCCTCTTCCTGAGCCCTG	60	451
Exon 3	Fw: 5'-CCATTGCACTCTAGCCCAGGTG Re: 5'-GGGAAGCCAGTAGGGGATGC	60	374
Exon 4 (coding region only)	Fw: 5'-CACTCAGGGCTGGAGCCTGG Re: 5'-GCCCTGGATAAGCCACCAGC	60	355

Table II

Oligonucleotides for luciferase activities of TXN2 promoter alleles

Allele	Oligonucleotides
A1	5'- GGGGGGGGACGTACATAA -3'
A2	5'- GGGGGGGGGA * ACGTACATAA -3''
A3	5'- GGGGGGGGAAACGTACATAA -3'
A4	5''- GGGGGGGGACGTACATAA -3'
A5	5'- GGGGGGGGGGGAACGTACATAA -3'

* The inserted nucleotides in different alleles were bolded.

Table III

Allele Analysis for TXN2 Polymorphism and Spina Bifida Risk: Odds Ratio Estimation by Logistic Regression

Alleles	No. of Control (%), N=96	No. Of Spina Bifida (%), N=96	OR (95% CI)
Non-Hispanic White			
A1	29 (48.3%)	25 (52.1%)	Reference
A2	28 (46.7%)	19 (39.6%)	0.8 (0.4–1.7)
Hispanic White			
A1	9 (45.0%)	14 (41.2%)	Reference
A2	8 (40.0%)	19 (55.9%)	1.5 (0.5–5.0)

A1 = Allele 1, ref; **A2** = Allele 2, GA insertion; **OR**: Odds Ratio; **95% CI**: Wald 95% Confidence Interval