Published in final edited form as:

Birth Defects Res A Clin Mol Teratol. 2015 September ; 103(9): 741–746. doi:10.1002/bdra.23343.

Heme Oxygenase-1 Promoter Polymorphisms and Risk of Spina Bifida

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Abstract

Background—Spina bifida is the most common form of neural tube defects (NTDs). Etiologies of NTDs are multifactorial, and oxidative stress is believed to play a key role in NTD development. Heme oxygenase (HO), the rate-limiting enzyme in heme degradation, has multiple protective properties including mediating antioxidant processes, making it an ideal candidate for study. The inducible HO isoform (HO-1) has two functional genetic polymorphisms: (GT)_n dinucleotide repeats and A(–413)T SNP (rs2071746), both of which can affect its promoter activity. However, no study has investigated a possible association between HO-1 genetic polymorphisms and risk of NTDs.

Methods—This case-control study included 152 spina bifida cases (all myelomeningoceles) and 148 non-malformed controls obtained from the California Birth Defects Monitoring Program reflecting births during 1990–99. Genetic polymorphisms were determined by PCR and AFLP/RFLP using genomic DNA extracted from archived newborn blood spots. Genotype and haplotype frequencies of two HO-1 promoter polymorphisms between cases and controls were compared.

Results—For (GT)_n dinucleotide repeat lengths and the A(–413)T SNP, no significant differences in allele frequencies or genotypes were found. Linkage disequilibrium was observed between the HO-1 polymorphisms (D' : 0.833); however, haplotype analyses did not show increased risk of spina bifida overall or by race/ethnicity.

Conclusion—Although, an association was not found between HO-1 polymorphisms and risk of spina bifida, we speculate that the combined effect of low HO-1 expression and exposures to known environmental oxidative stressors (low folate status or diabetes), may overwhelm antioxidant defenses and increase risk of NTDs and warrants further study.

Keywords

neural tube defects; spina bifida; heme oxygenase-1; genetic polymorphisms; oxidative stress

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Conflict of Interest: The authors have no conflicts of interest to declare.

Introduction

Spina bifida is a congenital disorder characterized by abnormal closure of the embryonic neural tube. It is the most common form of neural tube defects (NTDs), affecting 3 per 10,000 births annually in the United States (Center for Disease Control, 2014). Etiologies of NTDs are multifactorial (Martinez et al., 2009). Consistently observed risk factors for NTDs include: low maternal folate intake (Czeizel and Dudas, 1992), maternal obesity (Shaw et al., 1996), and maternal diabetes or prolonged hyperglycemia during pregnancy (Correa et al., 2008). Each of these is associated with increased oxidative stress (Hastie and Lappas, 2014; Niedzwiecki et al., 2014).

There is also a genetic component to the etiology of NTDs (Copp et al, 2013; Leck, 1974). Single nucleotide polymorphisms (SNPs) of genes involved in folate production have been studied extensively, including the 5, 10-methylenetetrahydrofolate (MTHFR), thymidylate synthetase (TYMS), dihydrofolate reductase (DHFR), and MTHF dehydrogenase (MTHFD1) (Molloy et al., 2009). However, these polymorphisms do not account for a large portion of NTD cases (Shaw et al., 2009), suggesting that alternative genes and pathways may be also involved.

We hypothesized that variants in genes functionally involved with defense mechanisms against oxidative stress may be associated with risk of NTDs. An ideal candidate to study is heme oxygenase (HO), the rate-limiting enzyme in heme degradation (Tenhunen et al., 1968), which has been hypothesized to play a role in myriad physiological and pathological conditions (Exner et al., 2004; Li et al., 2014; Maines, 1988). It has anti-inflammatory, anti-apoptotic, and antioxidant properties mediated through the equimolar production of its bioactive products carbon monoxide (CO) and bilirubin, respectively (Brouard et al., 2000; Stocker et al., 1987). In combination with another antioxidant defense mechanism, such as glutathione reductase, a body's redox status and homeostasis is finely regulated and redundant. In fact, an accumulation of the pro-oxidant heme, which occurs in individuals with deficiencies in HO-1 gene expression, can lead to further depletion of antioxidants. The gene of the inducible human HO isoform or HO-1 is mapped to chromosome 22q12 and a (GT)_n dinucleotide repeat microsatellite has been identified in the proximal promoter region (Alam et al., 2004). Length of the HO-1 (GT)_n microsatellite directly affects level of gene transcription (Chen et al., 2002; Exner et al., 2004). Longer repeats have higher risk of pulmonary, cardiovascular, and neurological diseases (Exner et al., 2004). Short (GT)_n repeat length increases the inducibility of HO-1 (Yamada et al., 2000). In addition, increased promoter activity has been associated with the A allele of A(−413)T SNP (rs2071746), which is located in the HO-1 genetic promoter regions close to (GT)_n dinucleotide repeat polymorphisms (Ono et al., 2004).

In this study, our objective was to investigate the association between functional polymorphisms in the HO-1 promoter and risk of spina bifida as no study, to our knowledge, has investigated this potential association.

Materials and Methods

STUDY DESIGN

In this case-control study, spina bifida cases and non-affected controls were obtained from the California Birth Defects Monitoring Program, an active ascertainment system whereby information on infants with or without birth defects was abstracted from multiple hospital reports and medical records following established procedures (Croen et al., 1991). Cases were infants with isolated spina bifida without other major birth defects. Non-malformed controls were randomly selected among all live births during the period of study, 1990–99.

Information on demographic characteristics for cases and controls was obtained from birth certificate files and included maternal age, sex, race/ethnicity, birthplace (US-born or non-US born), education level, and parity.

GENOTYPING

DNA was available from newborn screening dried blood spots obtained from linkage efforts made by the California Birth Defects Monitoring Program. Genomic DNA was extracted from bloodspots by using the protocol described by St. Julien et al. (2013). (GT)_n repeat lengths in the HO-1 promoter regions were amplified by polymerase chain reaction (PCR) and determined by amplified fragment length polymorphisms (AFLP), using the forward labeled primer; 5'-6-FAM-AGAGCCTGCAGCTTCTCAGA-3' and the reverse primer; 5'-ACAAAGTCTGGCCATAGGAC-3' (Yamada et al., 2000). The PCR reaction was performed in a total volume of 20 micro l, with the following amplification protocol: 95°C for 5/min, followed by 35 cycles of denaturation at 94°C for 30/s, annealing at 59°C for 30/s and extension at 72°C for 60/s. The PCR products were confirmed by gel electrophoresis in 2% agarose gels and visualized with ethidium bromide staining. The size of the labeled PCR product was determined with an ABI 3130xl Genetic Analyzer, using GeneScan® software (Applied Biosystems). The accuracy of the (GT)_n repeat determination by AFLP was verified by direct sequencing of the PCR product in each selected homozygote allele case ($n = 23, 24, 30$). We also genotyped the A(−413)T SNP, since this SNP has been reported to have a stronger effect on the HO-1 promoter activity than (GT)_n polymorphisms. For genotyping of A(−413)T SNP (rs2071746), we used PCR-RFLP method with a mismatched primer set (forward primer; 5'-GTTCCCTGATGTTGCCCCACCAAGCT-3'; and reverse primer; 5'-TCTGAGAAGCTGCAGGCTCTG-3') and Hind III restriction enzyme as described previously (Tiwari et al., 2013).

STATISTICAL ANALYSES

Demographic data were expressed as number (%) among cases and controls respectively. For both polymorphisms, we used HaploView (medical-and-population-genetics/haploview/haploview) (Barrett et al., 2005) to calculate MAFs (minor allele frequencies) and to evaluate deviations from Hardy-Weinberg equilibrium (HWE). To estimate associations between HO-1 polymorphisms and spina bifida risk, maximum likelihood estimates of the odds ratio (OR) and its corresponding 95% confidence intervals (CI) were calculated from logistic regression models using SAS (version 9.4), stratified by race/ethnicity. The association between a HO-1 polymorphism and spina bifida risk was assessed for

homozygotes of rare variants and heterozygotes by using wild-type homozygotes as referents. We also assessed association under a log-additive model of inheritance, where genotypes were treated as a quantitative variable (0 = 1/4 no less common allele, wild-type; 1 = 1/4 one less common allele, heterozygote; 2 = 1/4 two less common allele, homozygous mutant) and an OR was calculated for the increase of each less common allele. An OR with its 95% CI excluding 1 was considered statistically significant.

Haplotypes were constructed for the genes for each subgroup using HaploView. Haplotypes with a frequency of 0.1% or more were included. The OR of each haplotype was calculated using the sum of all other haplotypes as reference. Statistical significance was set at $p < 0.05$.

ETHICS REVIEW

This study was approved by the California State Committee for the Protection of Human Subjects and the Stanford University Institutional Review Board.

Results

The study included 152 spina bifida cases (all myelomeningoceles) and 148 controls. Univariate analysis showed statistically significant differences between cases and controls for maternal race/ethnicity, maternal education, and infant sex (Table 1).

Overall call rates and MAFs are summarized in Table 2. Observed genotype frequencies did not deviate from HWE (Table 2). The lengths of *HO-1* (GT)_n repeat in the study population were found to be highly polymorphic, ranging from 16 to 45, with a bimodal distribution with peaks at 23 and 30. Categorization of (GT)_n repeat by cases and controls yielded a similar distribution (Fig). We found no significant difference between average (GT)_n repeat length for cases and controls (cases; 29.5 (22–37.5), $n = 149$, controls; 29 (19.5–37.5), $n = 148$), 3 samples could not be genotyped due to poor DNA quality).

When the alleles were grouped into two allele subcategories: short (S): <26 (GT)_n repeats or long (L): ≥26 (GT)_n repeats, we found no significant difference in the frequencies of alleles (cases = S: 67; L: 231; controls = S: 75; L: 221) or genotypes (cases = SS: 3; SL: 61; and LL: 85; controls = SS: 9; SL: 57; and LL: 82) between cases and controls. Our results did not substantially change with stratification by race/ethnicity, although we found a higher frequency of SS/SL genotypes among non-Hispanic white cases (9/11) compared to controls (18/40), $p = 0.04$ (Table 3).

Because of limited quantities of extracted DNA, A(–413)T SNP genotyping could not be performed in 24 case samples and 17 control samples. We found no significant difference in the frequencies of alleles (cases = A: 161; T: 95; controls = A: 169; T: 93) or genotypes (cases = TT: 15; TA: 65; and AA: 48; controls = TT: 13; TA: 67; and AA: 51) between cases and controls. These results did not substantially change with stratification by race/ethnicity (Tables 3). Interestingly, we did observe that the OR from the additive model for the A(–413)T variant among whites was 2.1 and 2.2 for the (GT)_n variant among non-Hispanic whites.

Linkage disequilibrium (LD) was observed between *HO-1* (GT)_n repeat polymorphism and A(−413)T SNP (rs2071746) (D': 0.833; 95% CI: 0.73–0.90). Table 4 shows results from two-locus HO-1 haplotype analyses. These analyses did not show an increased risk of spina bifida overall or by race/ethnicity.

Discussion

In this study, we did not observe an elevated risk between spina bifida and HO-1 polymorphisms. We hypothesized that the antioxidant properties of HO-1 would modulate the risk of human spina bifida based on prior studies implicating a role of oxidative stress in the development of NTDs (Chang et al., 2003; Kase et al., 2013).

Although we found no risk association between functional HO-1 polymorphisms and spina bifida, it is possible that HO-1 polymorphisms amplify NTD development only among vulnerable patients, who already under additional sources of oxidative damage, such as insufficient maternal folate intake, obesity, or diabetes. We did not have information on these potential oxidative stressors to assess this hypothesis.

Maternal folic acid supplementation is associated with reduced risk of NTDs. Although the underlying mechanism of this therapy is not completely understood (Copp et al., 2013). We hypothesized a possible connection between folic acid supplementation and HO-1 based on the work of Saadeldien and others, who demonstrated that folic acid reduced oxidative damage from iron supplementation in neonatal rodent neuronal tissues (Saadeldien et al., 2012). Since we did not have data on folic acid supplementation in early pregnancy, the additional hypothesis about a modifying influence between folate and HO-1 variants could not be tested.

Pre-pregnancy obesity and pre-gestational diabetes are other known environmental contributors to NTDs. The pathologic mechanisms are unclear, but may be due to disturbances in glucose homeostasis during early pregnancy. Abnormal glucose homeostasis, in turn, could increase oxidative stress (Hastie and Lappas, 2014; Oliva et al., 2012). Yang and coworkers have also shown an increase in oxidative stress in neuronal tissues of the rodent embryos exposed to excess glucose levels (Yang et al., 1997). Furthermore, transgenic overexpression of free radical scavengers or supplementation of certain dietary antioxidants have been shown to reduce the rate of malformations in the offspring of diabetic animals (Hagay et al., 1995; Siman and Eriksson, 1997; Sivan et al., 1996). It is possible that a deficiency in antioxidant defense due to low HO-1 expression (due to long (GT)_n repeats or presence of the −413 T allele) in combination with an imbalance in redox homeostasis could further exacerbate fetal oxidative stresses of diabetic women (or those obese women with aberrant glucose control) and increase the risk of NTDs.

A strength of our study is that we investigated a novel genetic hypothesis in a population-based sample of cases and controls although the study was limited to inferences about the infant rather than maternal genotype. The study was also challenged by its modest size and lack of information on folic acid intake and blood glucose control parameters.

Although we did not find a risk association between HO-1 polymorphisms and spina bifida, the hypothesis we attempted to investigate here can be further studied by examining the effect of HO-1 polymorphisms in cases and controls, or their mothers, in the presence of known sources of environmental oxidative stressors, such as lack of folic acid or aberrant glucose control. Although we did observe a doubling in risk for the A(−413)T variant among non-Hispanic whites and for the (GT)_n variant among whites, this study was too small to provide statistically precise estimation of these associations. Thus, further investigations seem warranted, including those to assess the redox status of the most at-risk mothers.

Acknowledgments

We thank the California Department of Public Health, Maternal Child and Adolescent Health Division for providing data. The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the California Department of Public Health or the National Institutes of Health.

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Figure.

(GT)_n repeat length distribution. A bimodal (GT)_n repeat distribution with peaks at 23 and 30 were found. Categorization of (GT)_n repeat by cases and controls yielded a similar distribution.

Table 1

Characteristics of spina bifida cases and non-malformed controls, California 1990–99

Variable	Cases <i>n</i> = 152 (%) ^a	Controls <i>n</i> = 148 (%) ^a
Maternal Race/Ethnicity		
Non-Hispanic White	12 (7.9)	40 (27.0)
Hispanic	122 (80.3)	80 (54.1)
Black	8 (5.3)	12 (8.1)
Asian/Other	6 (4.0)	16 (10.8)
Maternal Age at Delivery (yrs)		
13–24	70 (46.1)	55 (37.2)
25–29	32 (21.1)	42 (28.4)
30–34	28 (18.4)	28 (18.9)
35–55	18 (11.8)	23 (15.5)
Maternal Education		
< High school	94 (61.8)	59 (39.9)
High school	23 (15.1)	36 (24.3)
> High school	32 (21.1)	53 (35.8)
Parity		
0	55 (36.2)	55 (37.2)
1	44 (29.0)	51 (34.5)
>1	50 (32.9)	41 (27.7)
Infant Sex		
Male	76 (50)	53 (35.8)
Female	76 (50)	95 (64.2)

^aPercentages may not equal to 100 due to missing data or rounding.

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Table 2
Characteristics of HO-1 promoter polymorphisms among spina bifida case and non-malformed control infants, 1990–99

SNP name	dbSNP ID	Position	Chromosome	Reference Allele ^a	Call Rate (%)	All Controls			White Controls			Hispanic Controls		
						MAF	HWE	p-value	MAF	HWE	p-value	MAF	HWE	p-value
A(−413)T	rs2071746	35776672	22	A	86	0.355	0.2681		0.365	0.8331		0.301		0.0831
(GT) _n repeat	rs71937010	35776838	22	L	99	0.253	1		0.262	1		0.219		0.4208

HWE: Hardy-Weinberg Equilibrium was evaluated using HaploView

Table 3

Genotype odds ratios (ORs) stratified by race/ethnicity

Label	SNP Name	dbSNP_ID	Genotype	Count (case, control)	OR (95% CI)
ALL	A(-413)T	rs2071746	TT	15, 13	1.2 (0.5–2.8)
			TA	65, 67	1.0 (0.6–1.7)
			AA	48, 51	Reference
			Additive Model ^b	128, 131	1.1 (0.7–1.6)
			TT/TA	80, 80	1.1 (0.6–1.8)
White	A(-413)T	rs2071746	TT	1, 4	3.5 (0.2–69.3)
			TA	8, 19	5.9 (0.7–52.7)
			AA	1, 14	Reference
			Additive Model ^b	10, 37	2.1 (0.6–6.6)
			TT/TA	9, 23	5.5 (0.6–48.0)
Hispanic	A(-413)T	rs2071746	TT	9, 3	2.2 (0.5–8.7)
			TA	50, 38	1.0 (0.5–1.8)
			AA	44, 32	Reference
			Additive Model ^b	103, 73	1.2 (0.7–1.9)
			TT/TA	59, 41	1.0 (0.6–1.9)
ALL	(GT)n	rs71937010	SS	3, 9	0.3 (0.1–1.2)
			SL	61, 57	1.0 (0.6–1.7)
			LL	85, 82	Reference
			Additive Model ^b	149, 148	0.8 (0.6–1.3)
			SS/SL	64, 66	0.9 (0.6–1.5)
White	(GT)n	rs71937010	SS	0, 3	N/A
			SL	9, 15	6.6 (1.2–34.9)
			LL	2, 22	Reference
			Additive Model ^b	11, 40	2.2 (0.7–6.8)
			SS/SL	9, 18	5.5 (1.1–28.8)
Hispanic	(GT)n	rs71937010	SS	2, 2	0.6 (0.1–4.6)
			SL	43, 31	0.9 (0.5–1.6)

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Label	SNP Name	dbSNP_ID	Genotype	Count (case, control)	OR (95% CI)
			LL	75, 47	Reference
			Additive Model ^b	120, 80	0.9 (0.5–1.4)
			SS/SL	45, 33	0.9 (0.5–1.5)

^aWald-test

^bORs measure increase in risk of each less common allele

Table 4

Haplotype analysis stratified by race/ethnicity

Race/ Ethnicity	Haplotype	Frequency	p-Value	Ratio Counts ^a (case, control)	OR (95% CI)
ALL	AL	0.613	0.6798	181.3 : 118.7, 183.8 : 112.2	0.9 (0.7–1.3)
ALL	TS	0.214	0.3509	59.5 : 240.5, 68.0 : 228.0	0.8 (0.6–1.2)
ALL	TL	0.148	0.1270	51.1 : 248.9, 37.2 : 258.8	1.4 (0.9–2.3)
ALL	AS	0.025	0.7926	8.1 : 291.9, 7.0 : 289.0	1.1 (0.4–3.2)
White	AL	0.59	0.2229	10.5 : 11.5, 49.7 : 30.3	0.6 (0.2–1.4)
White	TS	0.26	0.2482	7.8 : 14.2, 18.7 : 61.3	1.8 (0.7–5.0)
White	TL	0.116	0.9763	2.5 : 19.5, 9.3 : 70.7	1.0 (0.2–4.3)
White	AS	0.034	0.5723	1.2 : 20.8, 2.3 : 77.7	1.9 (0.2–18.7)
Hispanic	AL	0.654	0.5322	155.3 : 86.7, 107.5 : 52.5	0.9 (0.6–1.3)
Hispanic	TS	0.181	0.4469	40.9 : 201.1, 31.8 : 128.2	0.8 (0.5–1.4)
Hispanic	TL	0.141	0.1397	39.1 : 202.9, 17.5 : 142.5	1.6 (0.9–2.9)
Hispanic	AS	0.025	0.6227	6.7 : 235.3, 3.2 : 156.8	1.4 (0.4–5.4)

^aRatio counts: Estimated number of haplotype: estimated number of all other haplotypes in cases, estimated number of haplotype: estimated number of all other haplotypes in controls. Since the number of haplotypes cannot be directly counted from phase unknown genotyping data, estimated data (with decimal) is used in HaploView.