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Association of Retinoic Acid Receptor Genes with Meningomyelocele

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Abstract

BACKGROUND—Neural tube defects (NTDs) occur in as many as 0.5–2 per 1000 live births in the United ‘States. One of the most common and severe neural tube defects is meningomyelocele (MM) resulting from failed closure of the caudal end of the neural tube. MM has been induced by retinoic acid teratogenicity in rodent models. We hypothesized that genetic variants influencing retinoic acid (RA) induction via retinoic acid receptors (RARs) may be associated with risk for MM.

METHODS—We analyzed 47 single nucleotide polymorphisms (SNPs) that span across the three retinoic acid receptor genes using the SNPlex genotyping platform. Our cohort consisted of 610 MM families.

RESULTS—One variant in the *RARA* gene (rs12051734), three variants in the *RARB* gene (rs6799734, rs12630816, rs17016462), and a single variant in the *RARG* gene (rs3741434) were found to be statistically significant at $p < 0.05$.

CONCLUSION—RAR genes were associated with risk for MM. For all associated SNPs, the rare allele conferred a protective effect for MM susceptibility.

Keywords

retinoic acid receptor genes; meningomyocele; association studies; single nucleotide polymorphisms (SNPs)

INTRODUCTION

Neural tube defects (NTDs) are congenital defects that result from the malformation of the brain and spinal cord. NTDs are severely disabling and occur in 0.5–2 per 1000 pregnancies

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in the United States (Greene et al., 2009; Copp and Greene, 2010). One of the most common and severe among these neural tube defects is meningomyelocele (MM). MM results from failed closure of the caudal end of the neural tube during the first month of pregnancy. This incomplete formation results in exposure of the spinal cord and meninges (tissue covering the spinal cord). Surgical treatment to prevent infection and further damage to the spinal cord is available within the affected infant's first 24 to 48 hours of life. Despite corrective surgery, children with MM will likely need addition medical care, physical therapy, and remediation to overcome learning disabilities (Fletcher et al., 2005)

Although the exact cause remains unknown, the etiology of MM is linked to both genetic and environmental components. To better understand the pathophysiology of MM, rodent models have provided useful information (Juriloff and Harris, 2000; Harris and Juriloff, 2007). Vita-min A and its biologically active metabolite retinoic acid (RA) have been implicated in neuronal differentiation and neural tube patterning (Maden, 2006). Low dosage administration of RA has been found to reduce the incidence of spinal NTDs in mouse models (Wilson et al., 2003; Maden, 2006). Conversely, RA when administered in teratogenic doses has been known to induce MM in several different rodent species (Danzer et al., 2005; Harris and Juriloff, 2007; Quemelo et al., 2007). These observations suggest that MM susceptibility could be associated with the genes in the RA signaling pathways.

The effects of RA are mediated by two classes of ligand-dependent transcription factors, the retinoic acid receptors (RARs) and the retinoid X receptors (RARs). RARs are nuclear family receptor members that are bound and activated by both all-trans-RA and 9-cisRA, whereas only 9-cisRA activates RXRs (Mao and Collins, 2002; Zechel, 2005). Together RARs and RXRs interact to form heterodimers, later binding to RA response elements to regulate transcription of genes (Matt et al., 2003). There are three RAR genes, designated *RARA*, *RARB*, and *RARG*, each located on different chromosomes. With differing expression and localization patterns, it has been suggested that the three *RAR* genes are functionally distinct (Leroy et al., 1991). To further the complexity, *RARA* and *RARG* each have two major isoforms, whereas *RARB* has four distinct isoforms, resulting in a large potential of regulatory proteins and transcriptional functions.

We hypothesized that genetic variants influencing RA induction via RARs may be associated with risk for MM. We analyzed 47 single nucleotide polymorphisms that span across the three *RAR* genes and their association with MM.

MATERIALS AND METHODS

All experimental protocols were approved by the Committees for the Protection of Human Subjects at the University of Texas Health Science Center at Houston. Sites for subject enrollment included the Shriners Hospital for Children at Houston, Texas; the Shriners Hospital for Children at Los Angeles, California; the Shriners Hospital for Children at Lexington, Kentucky; Texas Children's Hospital, Houston, Texas; and the Hospital for Sick Kids, Toronto, Canada.

Patients with MM and their parents were enrolled in the study from 1996 to 2006 (Au et al., 2008). The tested samples comprised 329 affected family trios and 281 affected family duos. Trios included affected child and both parents, and duos included the affected child and only a single parent. Table 1 describes the ethnicity and sex of the probands tested. Ninety-two Hispanic individuals were recruited locally, and 92 Caucasian individuals were selected from the HD100CAU (Corriell Institute, Camden, NJ) to serve as genotyping controls.

DNA Collection and Preparation

Whole blood samples and/or saliva samples were collected from patients with MM and both parents when possible. Genomic DNA was extracted from blood using the Puregene DNA extraction Kit (Quiagen, Valencia, CA) and saliva using the Oragene DNA Collection Kit from DNA Genotek (Ontario, Canada).

SNPlex Genotyping

Single nucleotide polymorphism (SNP) genotyping was performed using the SNPlex Genotyping system (Applied Biosystems, Foster City, CA). The system is based on oligonucleotide ligation/polymerase chain reaction and capillary electrophoresis to simultaneously analyze up to 48 SNPs (Tobler et al., 2005). SNPs were selected (Table 2) and submitted through the SNPlex Assay Design pipeline to generate probe pools (Davidson et al., 2008; Martinez et al., 2009). Experiments were carried out according to ABI Standard SNPlex protocol. Two hundred nanograms of working DNA was used for each of the SNPlex reactions on Dual 384 well block ABI 9700s. Capillary electrophoresis of the SNPlex products was subsequently carried out on the ABI 3730 XL genetic analyzer. Data analysis was performed using GeneMapper version 4.0 software (Blue Heron, Bothell, WA). Genotype calls were evaluated independently by at least two investigators to ensure consistency.

Our criteria required SNPs to have at least an 85% genotype call rate. Genotypes were tested for Mendelian inheritance errors. SNPs with more than 1.6% (10/610) of families showing Mendelian errors were excluded (Martinez et al., 2009). To ensure SNPlex genotyping call accuracy, we included 84 CEPH individuals used in the Hapmap project. One hundred percent concordance was observed between the CEPH sample genotype results and genotypes published in the dbSNP NCBI database (GRCh37/hg19).

Statistical Analysis

Allele frequencies, Hardy-Weinberg equilibrium, and our triad-based association analyses were performed using a transmission disequilibrium test (TDT) in the PLINK v1.07 (http://pngu.mgh.harvard.edu/purcell/plink/) whole genome association analysis toolset (Purcell et al., 2007).

RESULTS

Of the 47 genotyped SNPs, two SNPs (one in *RARA* and one in *RARB*) failed to meet the quality control criteria and were excluded from further statistical analyses. Minor allele frequencies were calculated for the remaining 45 SNPs in Caucasian and Hispanic controls and are presented in Table 2.

Five SNPs were statistically significantly associated with MM ($p < 0.05$) (Table 3). All five statistically significant SNPs were in Hardy-Weinberg equilibrium, and none were in linkage disequilibrium when analyzed using Haploview software version 4.2 (Barrett et al., 2005). Even after correction for false positives by Bonferroni (1/45 expected false positives; $p < 0.022222$), all five SNPs remained significant.

Of the five SNPs tested in the *RARA* gene, only one (rs12051734) located in intron 2 of the longest transcription isoform (NM_000964.3) was found to be statistically significant with MM ($p = 0.01496$). Three SNPs (rs6799734, rs12630816, and rs17016462) of the 32 tested in the *RARB* gene demonstrated association with MM. Two of these SNPs, rs6799734 and rs17016462, are located in intron 1 and intron 2, respectively, of the longest transcription isoform (NM_000965.3) of *RARB*. The TDT analysis yielded a *p* value of 0.005459 for

rs6799734 and of 0.01954 for rs17016462. Rs12630816 ($p = 0.000546$) is a variant ~2 kbp upstream in the promoter region of *RARB*. Finally, among the eight SNPs tested in the *RARG* gene, only one (rs3741434) in the common 3′-untranslated region (3′-UTR) of all transcription isoforms was shown to be highly associated with MM ($p = 0.000246$). A summary of the five significant SNPs along with their gene location and *p* values is shown in Table 3.

DISCUSSION

To our knowledge, this is the first study to investigate association of *RAR* genes with MM in humans. In our study of 610 MM families, we identified five significant SNPs among the three *RAR* genes that demonstrate association with MM. All five SNPs that met our threshold for significance of $p < 0.05$ remained significant after Bonferroni testing for multiple comparisons. For all associated SNPs, the rare allele conferred a protective effect for MM susceptibility. Our findings are consistent with existing animal models that the *RAR* genes are associated with the development of MM.

The most significant result we found was that rs3741434, located in the 3′-UTR of both transcription isoforms of *RARG*, is significantly associated with MM risk. This finding, coupled with evidence from animal models, provides additional support that RA receptors, specifically *RARG*, could play a functional role to the etiology of MM.

Expression of *Rarg* during neural tube formation has been studied in the curly tail (ct) mouse, a classic model for human neural tube defects. Mouse mutants that are homozygous ct/ct develop spinal NTDs in more than 50% of cases (Maden, 2006). RA treatment at a specific time point in development prevents the ct/ct phenotype by enhancing posterior neuropore closure (Chen et al., 1994). In addition, in situ hybridization studies have shown that *Rarg* transcripts are deficient in the ct mutants and are up-regulated in the posterior neuropore following low-dose RA treatment (Chen et al., 1995).

Loss of *Cyp26A1* function is embryonic lethal and produces a phenotype that mimics RA-induced spina bifida (SB) (Abu-Abed et al., 2001). Cytochrome P450 (CYP26) enzymes are responsible for the inactivation of RA. The catabolic activity of CYP26A1 affects the available amount of RA throughout embryonic development. For this reason, Rat et al. (2006) screened coding sequences of the RA-metabolizing enzyme gene *CYP26A1* for mutation among 40 Italian SB patients and 40 healthy French “controls.” They found no evidence for involvement of *CYP26A1* in the pathogenesis of SB for their population. Their study was limited by small sample size and could have missed an association. Further, the finding does not surprise us because of the distinct functions of CYP26A1 (regulating the amount of RA available) versus RARs (transcription factors that activate RA responsive genes), nor does it weaken the conclusion of our results regarding the potential role of the RAR genes in MM susceptibility. When *Cyp26A1* null mutants are crossed with *Rarg* null mutants, the resulting double-knockout animals display normal gene expression patterns. Surprisingly, the lethal effects of the *Cyp26A1* null mutant are rescued when compounded with the loss of *Rarg* (Abu-Abed et al., 2003).

Another study (Deak et al., 2005) used the PCR sequencing method to screen coding sequences of five genes involving RA metabolism (*ALDH1A2*, *CYP26A1*, *CYP26B1*, *CRABP1*, and *CRABP2*) for mutation among 78 pooled samples of 230 SB patients and then genotyped 318 simplex and multiplex patient families for disease association analysis. The study concluded that several SNPs in the *ALDH1A2* gene associated with increased risk for lumbosacral myelomeningocele in humans. ALDH1A2 (also known as RALDH2) is the primary enzyme responsible for converting retinol to retinoic acid. However, the most

significant association was observed with a synonymous SNP (pA151A, c.453A>G) with a rare allele frequency that is extremely small, suggesting that the risk allele affects only a small portion of the patient population. The functional significance of the finding is not known. This is a larger study but does not directly address association of RARs to human MM. Our findings are consistent with the conclusion of this study.

The individual null *Rara*, *Rarb*, *Rarg* mice are viable and have shown mild to no phenotypic changes as a result of the ablations. Redundancy of the three RA receptors present in the genome may be a contributing factor for these observations. However, the *Rarg* null mutant when compared to the wild type shows complete resistance to RA-induced SB (Lohnes et al., 1993). In addition to the single null *Rar* mutants, double knockout mutants lacking two *Rar* isotypes have been studied, and these double mutants are embryonic lethal or postpartum lethal because of severe developmental defects (Mark et al., 2009).

In the largest of the three *RAR* genes evaluated, *RARB*, we identified three SNPs located in the promoter and introns 1 and 2 that show association with MM. The *RARB* gene is approximately 170 kbp long located at human chromosomal region 3q24.2. Twenty-three SNPs downstream spanning ~152 kbp of *RARB* were also evaluated, but no association was identified. In general, transcription regulatory sites are commonly located in the promoter and the first few introns of genes. Further experiments are needed to determine whether a specific allele of the associated SNPs affect transcription of *RARB*. The association of rs17016462 in intron 2 of *RARB* should be approached with caution due to its low MAF (0.02) within our Caucasian control group.

Rs12051734 associated with MM is located in intron 2 of the *RARA* gene. In silico analysis was performed on the significant SNPs using the Human Splicing Finder version 2.4 website to predict possible changes to mRNA splicing (Desmet et al., 2009). Within the *RARA* gene, when compared to the wild-type reference sequence, the T variant of intronic SNP rs12051734 was found to potentially disrupt splice site motifs. No other significant splicing predictions were found among the other four associated SNPs.

The strengths of our study include our relatively large cohort of 610 families, which is complemented by the use of the family-based TDT method. In addition, our sample set consists of two major affected ethnic groups: Hispanics and Caucasian, as well as two ethnically matched control groups. Among our 45 SNPs, 40 SNPs did not reach nominal significance. In addition to the five SNPs that were associated in our population as a whole, there were two SNPs (rs1465057 and rs6580936, both in the *RARG* gene) that were significantly associated only in our subset of Hispanic families and two other SNPs (rs1153594 and rs2715553 in the *RARB* and *RARA* genes, respectively) that were significantly associated only our subset of Caucasian American families. We view these results with caution because of the small sample size when separately analyzing the ethnic groups. A limitation is that the tested SNPs represent a small fraction of the known SNPs spanning the *RAR* genes.

Although we found SNPs among the three *RAR* genes to be associated with MM, the specific mechanism by which the *RAR* genes influence MM susceptibility remains unknown. MM is a complex disorder that occurs as a result of both genetic factors and the environment. To add further complexity, RA contribution to MM varies not only spatially, but also temporally. This study suggests that changes in RAR function may play a role in MM susceptibility. Further validation studies on an independently ascertained MM cohort, as well as functional investigation of the retinoic acid receptors, are warranted.

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Table 1

Study Population

Characteristic	Trios	Duos	Total
Ethnicity			
Caucasian	142	84	226
Hispanic	168	166	334
African American	4	14	18
Asian American	2	2	4
Other	7	5	12
Unreported	6	10	16
Total	329	281	610
Sex			
Male	155	129	284
Female	174	152	326

Table 2

Allele Frequencies for *RARA*, *RARB*, and *RARG* SNPs

Gene	SNP Name	Function	Allele1/ Allele 2	CAU[a]	HISP[b]
RARA	Rs2715554	Intron 2	C/T	0.13/0.87	0.52/0.48
	Rs12051734	Intron 2	C/T	0.89/0.11	0.84/0.16
	Rs2715553	Intron 2	C/T	0.48/0.52	0.25/0.75
	Rs4890109	Intron 4	G/T	0.95/0.05	1.00/0.00
RARB	Rs922939	Promoter	G/T	0.36/0.64	0.28/0.72
	Rs12630816	Promoter	C/T	0.90/0.10	0.87/0.13
	Rs1483856	Promoter	A/C	0.86/0.14	0.95/0.05
	rs7613553	Intron 1	A/C	0.43/0.57	0.20/0.80
	rs6799734	Intron 1	C/G	0.38/0.62	0.51/0.49
	rs13314219	Intron 1	A/C	0.52/0.48	0.49/0.51
	rs7629902	Intron 1	A/G	0.10/0.90	0.56/0.44
	rs6778350	Intron 2	A/G	0.12/0.88	0.28/0.72
	rs4681064	Intron 2	C/G	0.72/0.28	0.64/0.36
	rs17016462	Intron 2	A/T	0.98/0.02	0.91/0.09
	rs1368262	Intron 2	A/G	0.81/0.19	0.78/0.22
	rs6550982	Intron 2	A/G	0.38/0.62	0.51/0.49
	rs1286664	Intron 2	C/T	1.00/0.00	0.97/0.03
	rs1286648	Intron 2	A/C	0.52/0.48	0.44/0.56
	rs1368265	Intron 2	A/G	0.57/0.43	0.48/0.52
	rs17016566	Intron 3	C/G	0.92/0.08	0.91/0.09
	rs1881705	Intron 3	A/G	0.63/0.37	0.51/0.49
	rs1303629	Intron 3	G/T	0.20/0.80	0.45/0.55
	rs1153594	Intron 3	C/T	0.91/0.09	0.65/0.35
	rs1153597	Intron 3	C/T	0.56/0.44	0.36/0.64
	rs1153604	Intron 3	A/G	0.38/0.62	0.51/0.49
	rs1992005	Intron 3	C/T	0.88/0.12	0.84/0.16
	rs12638229	Intron 3	A/T	0.11/0.89	0.15/0.85
	rs1435706	Intron 3	A/T	0.33/0.67	0.38/0.62

Gene	SNP Name	Function	Allele1/ Allele 2	CAU[a]	HISP[b]
	rs1286765	Intron 3	C/T	0.46/0.54	0.26/0.74
	rs1286763	Intron 3	A/C	0.53/0.47	0.75/0.25
	rs7610827	Intron 3	A/T	0.33/0.67	0.38/0.62
	rs1730240	Intron 3	G/T	0.44/0.56	0.50/0.50
	rs3773445	Intron 4	C/T	0.54/0.46	0.59/0.41
	rs17526942	Intron 4	C/T	0.88/0.12	0.83/0.17
	rs1730230	Intron 4	G/T	0.10/0.90	0.14/0.86
	rs6782425	Intron 5	A/G	0.25/0.75	0.32/0.68
	rs2164360	Intron 5	C/T	0.75/0.25	0.82/0.18
RARG	rs7398676	Promoter	G/T	0.53/0.47	0.51/0.49
	rs11170488	Promoter	C/T	0.39/0.61	1.00/0.00
	rs7487904	5′-UTR	C/G	0.89/0.11	0.81/0.19
	rs10082776	Intron 2	A/G	0.90/0.10	0.78/0.22
	rs6580936	Intron 3	A/G	0.80/0.20	0.68/0.32
	rs1465057	Intron 3	C/T	0.10/0.90	0.19/0.81
	rs2229774	Ser427Leu	C/T	0.94/0.06	1.00/0.00
	rs3741434	3′-UTR	A/G	0.87/0.13	0.83/0.17

[a] Caucasian frequency A1/A2 (*N* = 145): 92 HD100CAU/53 CEPH.

[b] Hispanic frequency: A1/A2 Hispanics of Mexican American descent.

Function column interpreted with reference to sequences of the longest isoform of each gene: RARA, NM_000964.3; RARB, NM_000965.3; RARG, NM_000966.4.

Table 3

RAR Gene SNPs Associated with Meningomyocele ($p < 0.05$)

GENE	CHR	SNP	Minor allele	Common allele	T[a]	U[b]	Odds ratio	χ^2	TDT *p* value
RARA	17	rs12051734	T	C	61	91	0.6703	5.921	0.014960
RARB	3	rs12630816	T	C	17	44	0.3864	11.95	0.000546
RARB	3	rs6799734	C	G	79	118	0.6695	7.721	0.005459
RARB	3	rs17016462	T	A	18	35	0.5143	5.453	0.019540
RARG	12	rs3741434	G	A	50	94	0.5319	13.44	0.000246

[a] T: number of cases with minor allele transmitted.

[b] U: number of cases with minor allele not transmitted.