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Evaluation of GPR50, hMel-1B, and ROR-Alpha Melatonin-Related Receptors and the Etiology of Adolescent Idiopathic Scoliosis

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Abstract

Background—Adolescent idiopathic scoliosis (AIS) is the most common spinal deformity in children. Studies have shown low melatonin levels resulting from pinealectomy in chickens and mice result in the development scoliosis, while supplementation with melatonin after the pinealectomy prevented it. The mere characterization of low melatonin levels is not sufficient to explain the development of idiopathic scoliosis in primates and humans, but we hypothesize that a mutation in melatonin-related receptors may be involved with the development of scoliosis.

Methods—The coding, splice-site, and promoter regions of three melatonin-related receptors (hMel-1B, RORα, and GPR50) were evaluated by DNA sequencing for variants associated with the phenotype of adolescent idiopathic scoliosis. An initial screening of 50 scoliosis patients with adolescent idiopathic scoliosis was compared with 50 controls by DNA sequencing of the three receptors. Additional cases and controls were evaluated when genetic variants were observed (for a total of 885 individuals).

Results—No significant differences were found in the hMel-1B and RORα receptors. We found two cSNPs in GPR50 (rs561077 and rs13440581) in the initial 50 patients. To evaluate the significance of these cSNPs, an additional 356 patients and 429 controls were analyzed. When the combined groups were analyzed, no significant associations were observed.

Conclusions—Despite the observed relationship between melatonin and scoliosis, there is no significant association between mutations found in any known melatonin-related receptors with adolescent idiopathic scoliosis. The strong evidence of a melatonin-related cause for the development of idiopathic scoliosis still encourages research into undiscovered melatonin-related receptors, melatonin-related hormones, and the catalytic enzymes for the serotonin-melatonin pathway.

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Clinical Relevance—This investigation is a genetic testing of the remaining currently known melatonin-related receptors that have not previously been analyzed for association with AIS. Given the support in the literature of a relationship between melatonin and AIS, we have shown no mutations in any of the known melatonin-related receptor in patients with AIS.

Keywords

adolescent idiopathic scoliosis; melatonin; genetics

Introduction

Adolescent idiopathic scoliosis (AIS) is the most common spinal deformity in children. AIS is characterized by a 3-dimensional curvature of the spine in the absence of any congenital, neuromuscular, genetic, or other recognizable syndromes. Using 10° as the minimal degree of curvature required to make the diagnosis, the prevalence of AIS is 2-3% of the at-risk population (children 10-16 years of age). In addition, a severe curvature requiring treatment will generally develop in 1 out of 500 individuals, with a 10:1 greater frequency in females compared to males. While AIS is not a life-threatening disorder, its treatment is long, difficult, expensive, and associated with significant morbidity and complications. Despite extensive clinical and basic research, the cause of AIS remains unknown.[1]

A compelling hypothesis for the cause of AIS was derived from several reports demonstrating that experimental pinealectomies in newborn chickens (bipedal animals) lead to a spinal deformity similar to idiopathic scoliosis in humans.[2-10] The melatonin hypothesis for the genetic causation of adolescent idiopathic scoliosis originated in 1959, when Thillard first reported that pinealectomy produced scoliosis in chickens, which was confirmed by Dubousset *et al*.[2,4] Since the primary product of the pineal gland is melatonin, researchers investigated the melatonin levels after pinealectomies, and found that the chickens often developed scoliosis and very low melatonin levels.[4,5] Furthermore, transplantation of the excised pineal gland to the abdominal wall prevented the development of scoliosis in this animal model.

Several studies have investigated melatonin levels in patients with scoliosis and controls, but did not observe any significant differences; however, many studies have shown calmodulin levels are increased in patients with progressive scoliosis. It is possible that while the melatonin itself is normal, a melatonin or melatonin-related receptor could be mutated, which would interfere with melatonin's ability to interact with its transmembrane and intracellular receptors. Based on this possibility, the next logical step has been to propose that the causative agent for scoliosis exists not in melatonin itself, but rather some other component of the melatonin biochemical pathway, such as the melatonin-related receptors. In mammals, two subtypes of melatonin receptors have been isolated: melatonin 1A (hMel-1A) and melatonin 1B (hMel-1B) receptors. We have previously screened hMel-1A without any mutations found in the coding regions [21]. In addition, the RAR (retinoic acid receptor)-related orphan receptor (RORα) and G protein-coupled receptor 50 (GPR50) have been also associated with melatonin function. In this study, we performed a mutation analysis of the hMel-1B, ROR-α and GPR50 genes in patients with adolescent idiopathic scoliosis with the hypothesis that a mutation in melatonin-related receptors may be involved with the development of scoliosis.

Methods

Study Subjects and Ascertainment Strategy

All protocols were approved by the human subjects review board of the University of Iowa Hospitals and Clinics, Washington University in St Louis, Shriners Hospital, Texas Scottish Rite Hospital, and University of Colorado Denver. Informed consent was obtained from all

participants. Patients with AIS with a curve greater than 10° were identified through clinic lists and medical records databases. Patients with neuromuscular or congenital scoliosis or other recognizable syndromes involving scoliosis were excluded. In addition, patients with atypical features of scoliosis (left thoracic curves, very rapid progression, back pain, early onset, and neurological findings on clinical examination) were also excluded because of a possible association with intraspinal and brain stem abnormalities. The initial screening group from our institution utilized 50 patients with scoliosis and 50 unrelated control individuals. For the secondary evaluation, an additional 356 patients and 429 controls were recruited from the University of Iowa, Texas Scottish Rite Hospital for Children (Dallas), the University of Colorado (Denver), and Washington University (St. Louis).

DNA Extraction, PCR Amplification, and Direct Sequencing

Seven to 14 mL of whole blood was collected from each participant by venipuncture. DNA was extracted using a previously described protocol.[13] The concentration of each sample was approximately 20 ng/μL, and the samples were stored at 4°C. The mRNA sequences for hMel-1B (MTNR1B), RORα, and GPR50 were obtained through a nucleotide search on the NCBI website (http://www.nbci.nih.gov), and the complete cds were used. For hMel-1B (two exons), the mRNA was 1105 bp long, the accession number was NM_005959.3, and the version was NM_005959.3 GI:69122993. For GPR50 (two exons), the mRNA was 1939 bp long, the accession number was NM_004224, and the version was NM_004224.1 GI:4758467. For RORα (twelve standard exons and four alternative spliced exons), there are four different isoforms and variant 2 was used as the standard; the mRNA was 2020 bp long, the accession number was NM_134260, and the version was NM_134260.1 GI:19743900.

Primers were designed for each exon of hMel-1B, RORα, and GPR50 using the Primer3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The forward and reverse sequences and the product sizes produced by the designed primers are collected in Table 1. The primers were compared to the “hstg” database using BLAST search to ensure the primers annealed only where they were designed to anneal. After the primers were obtained, they were diluted to 20 μM and stored at -20°C.

The general recipe used for the PCR reactions per sample was 5 μL 10X Qiagen PCR Buffer, 1 μL dNTP (10mM each), 3 μL forward primer (20 μM), 3 μL reverse primer (20 μM), .5 μL *Taq* DNA polymerase (5 U/μL), 10 μL Q solution, 25 μL dH_2O, and 2.5 μL template DNA. The general cycling conditions used for the PCR reactions was activation of the *taq* polymerase at 95°C for 5 minutes and initial denaturation at 95°C for 2 minutes, 30 cycles were performed at 94°C for 1 minute, 57°C for 1 minute, and 72°C for 1 minute, followed by a last extension at 72°C for 10 minutes. The PCR products were purified using the QIAquick PCR Purification Kit (Cat. No. 28104) following the protocol enclosed in the kit. 20 μL of the purified DNA products were sent to the University of Iowa DNA facility for sequencing.

Sequence Analysis

After sequencing, the two sequences for each primer set were analyzed using both BLAT and BLAST searches. Both the positive and negative strands of each sample were simultaneously inputted into a BLAT search, and the percent homology and the position of the PCR amplification were recorded. Insertions, substitutions, deletions, and unknown nucleotides on one strand were checked against the other strand in a BLAST search. When a change was observed in the sequence of both strands, the change was labeled as a polymorphism. Finally, we searched the sequences of the control subjects at the locations polymorphisms were found and observed whether there were similar changes in the sequences of the control patients. The PLINK software package was used to perform Chi-Square Tests on the genotype and allele frequencies of the patients with AIS and the controls.[14]

Results

GPR50

By direct sequencing, we found two polymorphisms (rs561077 and rs13440581) in the second exon of the GPR50 receptor gene when scoliotic patient sequences were compared with the control sequence (accession number NM_004224). In the initial 50 patients screened, 66% of patients and 40% of controls had the variant allele at rs561077 (A-to-G), while 10% of patients and 0% of controls had the variant allele at rs13440581 (A-to-G). After we obtained the genotypes from the additional 356 patients and 429 controls, based on Pearson's Chi-Square Tests, the polymorphisms did not occur to a significantly higher degree in patients with AIS than in the controls ($P>.05$ for both polymorphisms). The patient population was in Hardy-Weinberg equilibrium. We also separated the total patient population based on the institution from which they were recruited, and performed the same chi-square analyses, but again no significant associations were found ($P>.05$). Finally, we ran a simple regression test using severity of curvature measured at the time of diagnosis as a continuous variable, but did not observe any significant association between genotype and severity.

hMel-1B and RORα

By direct sequencing of PCR-amplified DNA from this AIS study population, we found that the sequences of the different variant alleles for the hMel-1B receptor gene (accession number NM_005959.3) and the RAR (retinoic acid receptor)-related orphan receptor (RORα) (accession number NM_134260) were identical with the sequences obtained from our controls. No genetic variations were found for these two receptor genes; therefore, no significant correlation was determined between the hMel-1B and RORα receptors and the phenotype for AIS. In addition, we evaluated the Mel 1-B promoter SNP recently reported to be associated to AIS by Qiu *et al* in 180 patients with AIS and 180 controls, and found no significant difference in genotype frequencies.

Discussion

The melatonin hypothesis for the genetic causation of adolescent idiopathic scoliosis originated in 1959, when Thillard first reported that pinealectomy produced scoliosis in chickens, which was confirmed by Dubousset *et al*.[2,4] Since those early studies, the association between melatonin and the development of scoliosis has remained a promising, but contentious field of research. A critique of the evidence for the melatonin hypothesis is that simply the surgery required to perform a pinealectomy induces scoliosis, while the low melatonin levels are merely a secondary effect of the surgery and not directly associated with the development of scoliosis. Machida *et al* addressed this critique in a recent study where they induced experimental scoliosis in mice without pinealectomy and supplemented the scoliotic mice with melatonin.[15] Twenty-nine of 30 bipedal mice with no melatonin treatment developed scoliosis, 5 of 20 control quadrupedal mice developed scoliosis, but none of the bipedal mice with melatonin treatment developed scoliosis, which supported the contention that restoration of melatonin levels can prevent the development of scoliosis. Additionally, Machida *et al* designed a study that found pineal gland transplantation and melatonin supplementation following pinealectomy procedures prevented the development of spinal deformity.[16]

Several studies have proposed that melatonin-related receptors may be involved in scoliosis. Yong *et al* found that melatonin receptor expression in bilateral paravertebral muscles in AIS is asymmetric, but recommended further research to determine whether the asymmetry was a primary cause or secondary effect of scoliosis.[17] High-affinity melatonin receptors (hMel-1A and hMel-1B) have recently been cloned and appear to be the sites through which melatonin elicits its biologic effects.[18] The melatonin receptors localize in the hypothalamic

suprachiasmic nuclei, cerebellum and spinal cord, and nonneural sites including ovaries and blood vessels.[19] Also, these receptors may be associated with human balance control and sensorimotor performance.[20] Morcuende *et al* studied hMel-1A and found no mutations in the coding region of hMel-1A.[21] In this study we did not find any mutation in hMel-1B, which corroborates the study by Qiu *et al* which failed to identify a single nucleotide polymorphism associated with the occurrence of AIS.[22] A more recent study by Qiu *et al* found that a SNP in the promoter region of hMel-1B was associated with AIS.[23] We performed a screen of the SNP in 180 patients with AIS and 180 controls, and found no significant difference in genotype frequencies.

Recent research has also found evidence of a dysfunction in melatonin signaling pathway in patients with AIS. Moreau *et al* investigated the ability of melatonin to block cAMP accumulation in osteoblast samples collected from patients with AIS, and found this ability to be significantly depressed when compared with controls.[24] Later work by the same group showed evidence of a molecular interaction between hMel-1B and protein kinase C delta, and an improvement in the ability to suppress cAMP by melatonin in AIS osteoblasts when supplemented with estrogen.[25, 26] Hardeland *et al* recently provided an excellent review of melatonin, its receptors, and its signaling pathways.[27] In particular, the review provided a comprehensive discussion of the highly pleiotropic effects of melatonin that depend on the distribution and density of its receptors, G protein directed signaling through membranous receptors, and tissue-dependent melatonin metabolites. The intimate regulation of melatonin-related transcription factors and protein kinases by G-alpha variants, beta-gamma dimers, beta-arrestin, or ion channels provides an expanding area that demands further research in relation to AIS.

GPR50 is an X-linked, orphan G protein-coupled receptor that has been associated with melatonin-receptor regulation. Levoye *et al* found GPR50 heterodimerizes constitutively and specifically with hMel-1A and hMel-1B melatonin receptors, and abolished the high-affinity binding function of the hMel-1A melatonin receptor.[12] RORα is a nuclear hormone receptor that can bind to the hormone response elements of several genes as a possible enhancer, though the specific functions of this protein are not known. Wiesenberg *et al* proposed that RORα acts as a transcription factor that mediates nuclear melatonin signaling.[11]

In this study, we found two polymorphisms in the GPR50 receptor of scoliotic patients, but they were not present at a highly significant frequency when compared to the controls. The second exon of GPR50, in which the two cSNPs are found, encodes a transmembrane domain, which we would expect to be malfunctional in AIS patients. We initially hypothesized that the two polymorphisms in GPR50 could additively cause a severe conformational change in the protein structure of GPR50, thereby preventing GPR50 from properly binding to hMel-1A and hMel-1B and preventing GPR50 from abolishing the binding function of hMel-1A; however, this hypothesis was not supported by our data.

Several limitations of our study design should be addressed. First, we did not screen the non-coding (intronic) regions of GPR50, hMel-1B, and ROR-Alpha, and it is possible there may be a transcription binding site or enhancer region in the intronic sequence that could contain mutations. However, in genetic screens it is generally accepted not to evaluate the intronic regions given the excessive amount of DNA sequence information and the fact that the specific location of regulatory binding sites within the intronic regions are often unknown, as was the case in our genes. Second, we used relatively small patient and control populations in our initial screen, but our total population included over 400 patients and controls. Based on the initial sample size, it is possible that we missed rare mutations that cause a small proportion of scoliosis cases.

Overall, our results combined with the hMel-1A results of Morcuende *et al* demonstrate that no mutations in any known melatonin-related receptor are associated with the phenotype of AIS.[21] No significant differences were found in the GPR50, RORα, or hMel-1B coding, splice site, or promoter regions. The strong evidence of a melatonin-related cause for the development of idiopathic scoliosis still encourages research into undiscovered melatonin-related receptors, and hormones such as the melatonin-precursor serotonin, and the catalytic enzymes for the serotonin-melatonin pathway, HIOMT and NAT. The melatonin hypothesis remains a controversial, but promising field of research, and this study marks a significant finding in the understanding of the relationship between melatonin, melatonin receptors, and the development of adolescent idiopathic scoliosis.

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Table 1

Primer sequences and product sizes for hMel-1B, GPR50, and RORα exons.

Gene	Position	Forward Primer	Reverse Primer	Size (bp)
hMel-1B	Exon 1	GGGTCAGGGACAAGATCGA	AGCTGGGCAGGGAAGAGAG	391
hMel-1B	Exon 2	TGGCAAAGATCACAAACACC	TCTCGTGCTGACTGTTGCTC	596
hMel-1B	Exon 2	AGTTTGTCATGCTGCTGGTG	CCTATCGCTGTCGTGTCCTT	491
GPR50	Exon 1	attcggggagctcaaaaact	agagagggaggcacgcttt	380
GPR50	Exon 2	ggtccagatcctcacgtagc	cctttctccctccctcgata	482
GPR50	Exon 2	gcttagggtggccagagg	ttaccatcgtctgcatccac	594
GPR50	Exon 2	gtagtggcaggcttgggata	gaatgtccggaatgttccat	489
GPR50	Exon 2	tgcagtaaggcatctcactttg	ccaagtctgccttcagtgct	476
RORα	Exon 1	CACAGTGATCAGCAGAGCAAA	CATTCTTTGTGGCTGGGATT	376
RORα	Exon 2	AGGAGGCCTTTGGATTCACT	CAAGGCAGTGGCCAACTAAT	174
RORα	Exon 3	GGCTTCCCCTTTCTCTTCAT	GTTCAAGCATTTGGGGATGT	297
RORα	Exon 4	AGTGGAGACATACAAATCACAAAGA	TAACTGGTTGCGTGCTTCTG	186
RORα	Exon 5	CCCGTGCAACTGTACAACTC	CCACTCATTCTGACACTAACCTG	228
RORα	Exon 6	TGAATAGAGCATCCCAGGAGA	CAAAGCTCGCAGGAGAGAAT	504
RORα	Exon 7	CCTTCCCAATATTGCCTCAA	TGTGATCTCCCGTTTTCTCC	196
RORα	Exon 8	TCAAAATTCACTTGCAAAGCA	GGCATGCTTTGCTTTCATAAC	247
RORα	Exon 9	TTGTCCAAGCAGAGCTTTCA	ATGACCCAAATGACCCAAAC	229
RORα	Exon 10	CGTATGGGCCTTGTAAAAATTC	AAACTGTCTTTGTTTTCATTTTGG	211
RORα	Exon 11	ATAGTTCAGGCCATGCCAAC	CACCATGTCAGAGGAATGCT	199
RORα	Exon 12	TCAAGACAGAAAAAGGGTCCA	GGTTCCCTGATCTTTGATGC	473
RORα	ALT-SPL 1-1 (V11)	CCCCTCTCCAGCCTCCTAC	CTTTTTCCCTCCTGCTTTCG	351
RORα	ALT SPL 1-2 (V12)	GGTCACAGCAGCCAGACATA	ACTAAACGCCTTCCTGAGCA	200
RORα	ALT-SPL 2-3 (V32)	GCAGACAGACACTGACATGCT	GGGTTGACGCTGTTCTGTTT	233
RORα	ALT-SPL 4-1 (V41)	GGACCCAAGAGACTGACAGG	CTCTTATGGCTCGGGATGG	445

* The positions of the alternative splice sites for RORα are denoted by “ALT-SPL.”