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Deregulation of the planar cell polarity genes *CELSR3* and *FZD3* in Hirschsprung disease

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ABSTRACT

Hirschsprung disease (HSCR) is a congenital disorder characterized by the absence of intrinsic ganglion cells in the lower intestine. Genetic factors in the pathogenesis of this disease are under active investigation. As core genes in the planar cell polarity pathway, *Celsr3* and *Fzd3* are believed to play vital roles in the development of the murine enteric nervous system. The potential association of *CELSR3* and *FZD3* with the development of HSCR in humans, however, is still unknown. We determined the genotypes of eight *CELSR3* and *FZD3* polymorphisms in 113 patients. Furthermore, target gene sequencing was used to search for rare mutations in the planar cell polarity genes. The mRNA and protein expression of *CELSR3* and *FZD3* were explored in patients with HSCR. Class III β-tubulin in colon tissue samples was examined to elucidate enteric innervation patterns. We observed a significant association between the *FZD3* rs17059206 polymorphism and HSCR susceptibility ($p < 0.001$). In addition, five rare mutations in *CELSR3* were identified in six patients with HSCR. Upregulation of *CELSR3* mRNA expression was detected in 80% of aganglionic segments; a similar increase was found for *FZD3* protein expression in 81.8% of aganglionic tissues, compared with the ganglionic segments. Immunohistochemical staining on tissue sections revealed obvious excess expression of both molecules in the mucosal layer. The neurite patterns were highly disorganized in the aganglionic bowel segments, with a marked reduction in the prominence of TUJ1 bundles in number, thickness, and length. Our results showed that deregulation of the planar cell polarity genes *CELSR3* and *FZD3* might disrupt the enteric innervation pattern and consequently contribute to the susceptibility to HSCR.

1. Introduction

The enteric nervous system (ENS) is part of the peripheral nervous system that controls functions of the intestine largely independent of the central nervous system (CNS) [1]. Many properties of the ENS resemble those of the CNS, such as exhibiting a wide array of neuronal and glial cells and releasing the same neurotransmitter [2]. Disturbance of one or more of the ENS functions can lead to congenital or acquired enteric neuropathies including Hirschsprung disease (HSCR), hypoganglionosis, and neuronal intestinal dysplasia. While these disorders have been recognized for several decades, the underlying genetic pathophysiology has not been resolved.

HSCR is a congenital disorder of the ENS, characterized pathologically by a lack of enteric ganglia in the myenteric and submucosal plexuses. This aganglionosis, resulting from incomplete rostral to caudal enteric colonization, affects variable lengths of the intestine [3]. The disease has an incidence of about 15/100,000 live births in newborns of European populations and twice that among Asian neonates. About 20% of the cases are familial, while the vast majority (80%) are sporadic ones showing a complex non-Mendelian mode of inheritance, high heritability (>80%), significant sex differences (male to female 4:1), and a high sibling recurrence risk (4%) [4]. Genetic studies have identified rare coding, high-penetrance mutations in 14 genes that mainly explain the pathogenesis of syndromic or familial cases. Discovery of these genes has revealed critical ENS-related developmental pathways; however, these pathogenic alleles cumulatively occur in <20% of cases, and the genetic causality for the remainder is unknown [5–7].

The planar cell polarity (PCP) pathway is evolutionarily conserved in vertebrates and is important for disparate processes such as gastrulation and neuron orientation [8]. In mammals, PCP-dependent processes include neuronal migration, axon growth, and the axon guidance system [9]. Sasselli et al. recently discovered that PCP genes play important

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roles in gastrointestinal development by regulating the spatial organization of the primary neural processes of nascent enteric neurons during murine embryogenesis [10]. In mice, *Celsr3* and *Frizzled3* (*Fzd3*), members of the "core PCP genes", control a nearly identical set of axon growth and guidance processes. Loss-of-function mutations in either gene lead to severe abnormalities in the anterior commissure and disturbed development of the major fiber tracts such as the thalamocortical, corticothalamic, and nigrostriatal tracts [11–13]. A growing number of studies have revealed additional defects in *Celsr3* and *Fzd3* homozygous mutant mice, including failure of cranial motor axons to reach their targets, stalling of spinal motor axons that are intended to innervate the dorsal limb, and defective migration of a subset of neural crest cells [14]. *Fzd3* is essential for the development of sympathetic chain ganglia and innervation of the sympathetic targets in mice [15]. Intriguingly, the PCP signaling genes *Celsr3* and *Fzd3* are required to control the guidance and growth of enteric neuronal projections relating to the longitudinal and radial axes of the intestine. Deletion of these genes disrupts the normal development of neuronal projections in newborns, leading to subtle changes in neurites of the mature ENS, resulting in obvious gastrointestinal movement abnormalities in mice [10]. Based on these studies, we raised the hypothesis that *CELSR3* and *FZD3* may participate in the development of the human ENS, and their aberrant expression, distribution or function may contribute to an increased risk for Hirschsprung disease.

2. Material and methods

2.1. Patients and specimens

This study was approved by the Ethics Committee of the Capital Institute of Pediatrics (Ethical Number: SHERLL 2013039). Blood samples of 133 patients with sporadic HSCR (61 short-segment, 19 long-segment, 35 total colonic aganglionosis, 18 unknown), with patients' ages ranging from 5 days to 13 years, including 95 male and 38 female individuals, were collected from the Department of General Surgery, Capital Institute of Pediatrics. Among these, 113 patient samples were used for genotype analysis and 82 for target gene sequencing. An additional 115 healthy children that matched the 113 patients with HSCR in age and sex were used as controls. Both normal and stenotic tissue samples were obtained from 22 patients with HSCR (sixteen short-segment, six long-segment). The patients ranged from 19 days to 5.8 years of age, and included 17 male and five female individuals. Based on the results of intestinal morphology examination during and after the operation, the surgical specimens were divided into normal and stenotic colon segments, which were further confirmed by histological examination of a frozen section of each segment. The aganglionic colon segments were defined by the absence of focal colonic ganglion cells by hematoxylin and eosin staining.

2.2. Genomic DNA extraction

Venous blood (1–2 mL) was obtained from the study participants using EDTA as an anticoagulant. Genomic DNA of peripheral blood leukocytes was extracted according to the simple salting-out method [16].

2.3. Genotype examination

Eight common tag single nucleotide polymorphisms (SNPs) were selected based on their minor allele frequency (MAF > 0.05) in the Chinese Han Beijing, northern Chinese population and the choice provided by the Tagger pairwise tagging system from the 1000 Genome Project (http://www.1000genomes.org/1000-genomes-browsers). The r^2 cut off value used to pick the tagging SNPs is 0.8. There are 70 and 389 common SNPs (MAF > 0.05) present at the *CLESR3* (Chr3: 48673896-48700348, 26 kb) and *FZD3* (Chr8:28351722-28431785, 80 kb) locus respectively, from which the tagging SNPs were finally selected. The SNPs were examined using TaqMan® human genotyping assays following the manufacturer's protocol (Applied Biosystems, USA). Specifically, the genotypes of five *CELSR3* SNPs: rs9868809 (assay ID: C__29828589_10), rs2286652 (assay ID: C___2649618_1_), rs2302295 (assay ID: C__2965557_1_), rs6773261 (assay ID: C___2965565_10_), and rs3821875 (assay ID: C___2965555_20) and three *FZD3* SNPs: rs3735725 (assay ID: C__27471132_20), rs12679661 (assay ID: C__30776136_10), and rs17059206 (assay ID: C__32588108_10) were examined. In brief, quantitative PCR (qPCR) protocols were run in a final volume of 10 μL in a 96-well plate, including 5 μL of 2× TaqMan® Genotyping Master Mix, 0.25 μL of 40× Assay Mix, 2.75 μL of deionized water, and 20 ng of DNA template. Reactions were set up on an Applied Biosystems 7500 Fast real-time PCR detection system using the following thermocycling conditions: 95 °C/10 min, then 92 °C/15 s 60 °C/60 s for 40 cycles and analyzed with the TaqMan® Genotyper software program. All SNPs had call rates >94.7%.

2.4. Target gene sequencing

We applied the next-generation target gene sequencing system to identify rare variants (MAF < 0.01 in the general population) from the pooled DNA of patients with HSCR. The core genes of the PCP signaling pathway (*CELSR3, FZD3, VANGL1, VANGL2, PRICKLE1, PRICKLE2, DVL1, and DVL2*) were enriched by a gene capture strategy using the GenCap Custom Enrichment Kit (MyGenostics, Beijing, China) according to previously described methods [17]. Briefly, a minimum of 3 μg of DNA was used for the indexed Illumina libraries construction following the manufacturer's protocol (Illumina, San Diego, CA). A final library size of 350–400 bp, including adapter sequences, was selected. Next, 1 μg of the DNA library was mixed with Buffer BL and a GenCap hypercholesterolemia probe (MyGenostics), then heated in a PCR instrument at 95 °C for 7 min and 65 °C for 2 min. For hybridization, 23 μL of prewarmed (65 °C) Buffer HY (MyGenostics) was added, and the mixture was held at 65 °C with the PCR lid heat on for 22 h. Three washes followed, using 50 μL of Dynabeads MyOne beads (Life Technologies, Carlsbad, CA) in 500 μL of 1× binding buffer and resuspended in 80 μL of 1× binding buffer. Then, 64 μL of 2× binding buffer was added, and the mixture was transferred to a tube containing 80 μL of MyOne beads and spun for 1 h on a rotator. The beads were then washed once with WB1 buffer at room temperature for 15 min and WB3 buffer (Life Technologies) three times at 65 °C for 15 min. Elution buffer was used to elute the bound DNA, which was amplified as follows: 98 °C for 30 s, 98 °C for 25 s, 65 °C for 30 s, 72 °C for 30 s (15 cycles), 72 °C for 5 min. We purified the PCR product using solid-phase reversible immobilization beads (Beckman Coulter) following the manufacturer's protocol. Enrichment libraries were sequenced on an Illumina HiSeq 2000 sequencer (Illumina) for 100-bp paired reads.

After sequencing, we retrieved high-quality reads from raw reads by filtering out low-quality reads (mapping qualities < 30, Total Mapping Quality Zero Reads < 4, long homo-polymer run > 5, approximate read depth < 5, QUAL < 50.0, phred-scaled *p*-value using Fisher's exact test to detect strand bias > 10.0) and adapter sequences using SolexaQA software [18] and the cutadapt program, respectively. We used the SOAPaligner program [19] to align the clean read sequences with the human reference genome (http://genome.ucsc.edu/). After removing the duplicates with Picard software [20], SNPs were identified using the SOAPsnp program [19] (http://soap.genomics.org.cn/). Subsequently, reads were realigned to the reference genome using the Burrows–Wheeler alignment program [21], and insertions or deletions (InDels) were identified with the Genome Analysis Toolkit [22] (http://www.broadinstitute.org/gsa/wiki/index.php/Home_Page). We annotated the identified SNPs and InDels with the exome-assistant program. Short read alignment, candidate SNP, and InDel validation were performed using MagicViewer [23]. We used the SIFT, PolyPhen, and MutationTaster algorithms to evaluate nonsynonymous variants to determine their pathogenicity.