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Mutations in *RAD21* Disrupt Regulation of APOB in Patients With Chronic Intestinal Pseudo-Obstruction

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BACKGROUND & AIMS: Chronic intestinal pseudo-obstruction (CIPO) is characterized by severe intestinal dysmotility that mimics a mechanical subocclusion with no evidence of gut obstruction. We searched for genetic variants associated with CIPO to increase our understanding of its pathogenesis and to identify potential biomarkers. **METHODS:** We performed whole-exome sequencing of genomic DNA from patients with familial CIPO syndrome. Blood and lymphoblastoid cells were collected from patients and controls (individuals without CIPO); levels of messenger RNA (mRNA) and proteins were analyzed by quantitative reverse-transcription polymerase chain reaction, immunoblot, and mobility shift assays. Complementary DNAs were transfected into HEK293 cells. Expression of *rad21* was suppressed in zebrafish embryos using a splice-blocking morpholino (*rad21a*). Gut tissues were collected and analyzed. **RESULTS:** We identified a homozygous mutation (p.622, encodes Ala>Thr) in *RAD21* in patients from a consanguineous family with CIPO. Expression of *RUNX1*, a target of RAD21, was reduced in cells from patients with CIPO compared with controls. In zebrafish, suppression of *rad21a* reduced expression of *runx1*; this phenotype was corrected by injection of human *RAD21* mRNA, but not with the mRNA from the mutated p.622 allele. *rad21a* Morpholino zebrafish had delayed intestinal transit and greatly reduced numbers of enteric neurons, similar to patients with CIPO. This defect was greater in zebrafish with suppressed expression of *ret* and *rad21*, indicating their interaction in the regulation of gut neurogenesis. The promoter region of *APOB* bound RAD21 but not RAD21 p.622 Ala>Thr; expression of wild-type RAD21 in HEK293 cells repressed expression of *APOB*, compared with control vector. The gut-specific isoform of APOB (APOB48) is overexpressed in sera from patients with CIPO who carry the RAD21 mutation. APOB48 also is overexpressed in sporadic CIPO in sera and gut biopsy specimens. **CONCLUSIONS:** Some patients with CIPO carry mutations in *RAD21* that disrupt the ability of its product to regulate genes such as *RUNX1* and *APOB*. Reduced expression of *rad21* in zebrafish, and dysregulation of these target genes, disrupts intestinal transit and the development of enteric neurons.

Keywords: Sporadic and Familial Chronic Intestinal Pseudo-obstruction; Intestinal Motility; Animal Model; Genetic Analysis.

Chronic intestinal pseudo-obstruction (CIPO), a rare and potentially life-threatening disorder with unknown prevalence and incidence,[1–3] is viewed typically as an insufficiency of the intestinal peristalsis that mimics a subocclusive disease in the absence of mechanical obstructions.[4–6] The severity of the clinical presentation and the limited understanding of the disorder contribute to poor quality of life and increased mortality.[2,4–7] In addition, there are no specific biochemical or molecular biomarkers of CIPO, further hindering a correct diagnosis. From a genetic perspective, most patients with CIPO experience sporadic occurrences, although X-linked, autosomal-dominant, and recessive forms have been identified with mutations in filamin A,[8,9] actin G2,[10] thymidine phosphorylase[11]/polymerase γ,[12] and, more recently, in *SGOL1*.[13] However, the underlying genetic alterations and molecular mechanisms remain unknown in most CIPO cases.

We previously mapped a locus in a large consanguineous family, segregating an autosomal-recessive form of CIPO.[14,15] In the affected family members, the major clinical feature was represented by CIPO, in addition to megaduodenum,

Abbreviations used in this paper: **APOB, apolipoprotein B; cDNA, complementary DNA; CIPO, chronic intestinal pseudo-obstruction; dpf, days postfertilization; IBS, irritable bowel disease; LCL, lymphoblastoid cell line; MO, morpholino; mRNA, messenger RNA; RT-PCR, reverse-transcription polymerase chain reaction; SNP, single-nucleotide polymorphism.**

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long-segment Barrett esophagus, and cardiac abnormalities of variable severity (OMIM 611376; Mungan syndrome). Here, we intersected mapping data with whole-exome sequencing to identify *RAD21* as a causal locus for CIPO. Our combined genetic and functional data suggest a loss-of-function mechanism that disrupts the structure and function of enteric innervation. Moreover, based on our previous observations that identified apolipoprotein B (APOB) as a target of RET signaling,[16] we explored the role of this protein in CIPO etiopathology in the context of RAD21 mutations. Here, we report a key role for APOB48, a gut-specific isoform,[17] as a transcriptional target of RAD21, and thus a contributor to CIPO, with potential utility as a biomarker.

Materials and Methods

Patients and Controls

The clinical characteristics of the patients with syndromic CIPO are indicated in the Supplementary Material and Methods section. An additional 21 Italian and 12 Swedish sporadic patients with idiopathic CIPO were included in the study (8 men and 25 women; mean age, 38.6 ± 16.6 y). Table 1 shows the major clinical characteristics of these patients. Five hundred Turkish controls were recruited at the Universities of Ankara and Istanbul; 240 controls of European ancestry were recruited at the University of Bologna. All data from patients and controls, including informed consent, were handled in accordance with local ethical committee–approved protocols and in compliance with the Helsinki declaration (http://snp.gs.washington.edu/SeattleSeqAnnotation137).

High-Throughput Single-Nucleotide Polymorphism Genotyping and Whole-Exome Sequencing Analysis

A detailed description of the single-nucleotide polymorphism (SNP) genotyping and whole-exome sequencing analysis is reported in the Supplementary materials. Variant detection and genotyping were annotated with the SeattleSeq137 Annotation Server.

RAD21 Mutation Screening in Idiopathic CIPO Cases

Genomic DNA extracted from peripheral blood was amplified as reported in the Supplementary materials.

RAD21 Complementary DNA Transfection Into HEK293 Cells

HEK293 cells (3×10^5) were plated for transfection of the different plasmids using liposomes as described in the Supplementary materials.

Gene Expression Analysis

Total RNA from 1.5 mL fresh blood was extracted with the Qiagen Blood Total RNA kit (Qiagen, Venlo, Limburg, The Netherlands). Total RNA from lymphoblastoid or transfected cells was extracted with the RNeasy kit (Qiagen). Real-time quantitative reverse-transcription polymerase chain reaction (RT-PCR) was performed as reported in the Supplementary materials.

Zebrafish Functional Assays

To determine the effect of *rad21a* suppression in zebrafish embryos, a splice-blocking morpholino (MO) was designed as described in the Supplementary materials. A published *ret* MO was used.[18] To measure *rad21a* MO efficiency, total messenger RNA (mRNA) was extracted from control and MO-injected embryos, reverse-transcribed, and the site targeted by the MO was PCR-amplified (Supplementary Figure 1). *runx1* expression analysis and enteric nervous system characterization are reported in the Supplementary materials.

Microgavage

Control and rad21 MO-injected embryos were developed until 5 days postfertilization (dpf). Zebrafish larvae were anesthetized in Tricaine (Sigma, St. Louis, MO), mounted in 3% methylcellulose, and injected with fluorescent beads into the mouth as described.[19]

Electromobility Shift Assay

LCLs (2×10^6) were processed for nuclear extract preparation as described in the Supplementary materials.

Immunoprecipitation and Western Blotting

Lymphoblastoid cell lines (LCLs) (2×10^6) were used for immunoprecipitation assays. Crude sera of patients was diluted in phosphate-buffered saline. Serial dilutions for cases and controls were performed (1:5, 1:10, and 1:100) (Supplementary Figure 2*A*). Immunoprecipitation and Western blotting were performed as reported in the Supplementary materials.

Immunohistochemistry

Immunohistochemistry was performed as reported in the Supplementary materials. Incubation with the corresponding blocking peptides or with the secondary antibodies only were performed as negative controls (Supplementary Figure 2*B* and *C*).

Quantitative Evaluation of Ganglion Cells

Quantitative evaluation of neuron number in myenteric and submucosal ganglia was performed according to Ganns et al[20] (Supplementary materials).

Statistical Analysis

A case-control association study for SNP rs72105712 was performed using Haploview 4.0 (http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/). A statistical analysis of quantitative differences was performed using the Student *t* test from the GraphPad package (http://graphpad.com/quickcalcs/). A fluorescent cell count was performed with ImageJ (National Institutes of Health, Bethesda, MD) (http://rsbweb.nih.gov/ij); chi-squared tests were calculated using the dedicated option from GraphPad.

Results

Identification of a Novel RAD21 Mutation in CIPO

We performed a combined SNP-genotyping/next generation-sequencing approach in a consanguineous