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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Mutations in Plasmalemma Vesicle Associated Protein Result in Sieving Protein-Losing Enteropathy Characterized by Hypoproteinemia, Hypoalbuminemia, and Hypertriglyceridemia

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SUMMARY

This study describes a novel form of severe fatal Protein Losing Enteropathy caused by a nonsense mutation in Plasmalemma Vesicle Associated Protein (PLVAP) gene resulting in loss of PLVAP mRNA and protein expression of fenestrae diaphragms and compromised endothelial barrier function.

BACKGROUND & AIMS: Severe intestinal diseases observed in very young children are often the result of monogenic defects. We used whole-exome sequencing (WES) to examine genetics in a patient with a distinct severe form of protein-losing enteropathy (PLE) characterized by hypoproteinemia, hypoalbuminemia, and hypertriglyceridemia.

METHODS: WES was performed at the Centre for Applied Genomics, Hospital for Sick Children, Toronto, Canada, and exome library preparation was performed with the Ion Torrent AmpliSeq RDY Exome Kit. Functional studies were based on the identified mutation.

RESULTS: Using WES we identified a homozygous nonsense mutation (1072C>T; p.Arg358*) in the *PLVAP* (plasmalemma vesicle-associated protein) gene in an infant from consanguineous parents who died at 5 months of age of severe PLE. Functional studies determined that the mutated PLVAP mRNA and protein were not expressed in the patient biopsy tissues, presumably secondary to nonsense-mediated mRNA decay. Pathological analysis showed that the loss of PLVAP resulted in disruption of endothelial fenestrated diaphragms.

CONCLUSIONS: The PLVAP p.Arg358* mutation resulted in the loss of PLVAP expression with subsequent deletion of the diaphragms of endothelial fenestrae, which led to plasma protein extravasation, PLE, and ultimately death. *(Cell Mol Gastroenterol Hepatol 2015;1:381–394; http://dx.doi.org/10.1016/j.jcmgh.2015.05.001)*

Keywords: Endothelium; Fenestrae; Hypertriglyceridemia; Hypoalbuminemia; Hypoproteinemia; Very Early Onset Inflammatory Bowel Disease; Monogenic Diseases; Protein-Losing Enteropathy; Whole-Exome Sequencing.

Protein-losing enteropathy (PLE) is characterized by excessive loss of protein often due to the disruption of the integrity of the intestinal mucosal membrane or dilatation of the intestinal lymphatic system. Two broad categories of PLE have been described: mucosal injury causing the excessive losses observed in inflammatory bowel disease (IBD) and intestinal infections, and abnormalities of the lymphatic system observed in primary intestinal lymphangiectasia.[1,2] The latter encompasses the group of patients who present with hypoalbuminemia, edema, and dilatation of the lymphatics of the enteric system of unclear etiology.

Recently there has been growing interest into the genetic causes of severe intestinal phenotypes.[3] For example, a novel

*InterNational Early Onset Pediatrics IBD Cohort Study (www.NEOPICS.org). §Authors contributed equally. ‡Authors contributed equally.

Abbreviations used in this paper: BSA, bovine serum albumin; DPBS, Dulbecco's phosphate-buffered saline; EpCAM, epithelial cell adhesion molecule; HA, human influenza hemagglutinin; hrGFP, humanized *Renilla* green fluorescent protein; IBD, inflammatory bowel disease; PAS, periodic acid–Schiff; PBS, phosphate-buffered saline; PLE, protein-losing enteropathy; PLVAP, plasmalemma vesicle-associated protein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TEM, transmission electron microscopy; VEOIBD, very early onset inflammatory bowel disease; VLDL, very-low-density lipoprotein; PCR, polymerase chain reaction; WES, Whole-Exome Sequencing.

2352-345X
http://dx.doi.org/10.1016/j.jcmgh.2015.05.001

Mendelian form of apoptotic enterocolitis caused by mutations in *TTC7A* was recently reported.[4] However, in many infants with severe intestinal disease, including PLE, the causative genetic defects have yet to be identified.[3] Here we use whole-exome sequencing (WES) to identify a nonsense mutation in the plasmalemma vesicle-associated protein (*PLVAP*) gene that results in a distinct severe form of PLE characterized by hypoproteinemia, hypoalbuminemia, and hypertriglyceridemia. The human form of PLVAP deficiency is nearly identical to that observed in *Plvap* knockout mice,[5] demonstrating the critical role of PLVAP in endothelial barrier function and intestinal homeostasis.

Materials and Methods

Patients

All experiments were performed with the approval of the research ethics board at the Hospital for Sick Children. Informed consent to participate in research was obtained. A copy of the consent is available on the website of the International Early Onset Paediatric IBD Cohort Study (NEOPICS) at http://www.neopics.org/NEOPICS_Documents.html.

Samples from our patient with the PLVAP p.Arg358* mutation were obtained on two occasions during endoscopic investigation for severe PLE. Control samples from the duodenum or colon were obtained from patients who were undergoing evaluation of gastrointestinal symptoms, among whom the endoscopic, histologic, and follow-up clinical impressions were normal. A case of congenital tufting enteropathy as well as microvillus inclusion disease initially presenting with PLE were assigned as duodenal disease controls. Biopsies from a patient with IBD with inflamed areas in the colon served as a colonic disease control.

Next-Generation Sequencing

WES was performed at the Centre for Applied Genomics, Hospital for Sick Children, Toronto, Canada. The exome library preparation was performed using the Ion Torrent AmpliSeq RDY Exome Kit (Life Technologies, Carlsbad, CA) following the manufacturer's recommended protocol. In brief, 100 ng of DNA quantified by Qubit DNA HS or BR assay (Life Technologies) was used in the target amplification under the following conditions: 99°C for 2 minutes, followed by 10 cycles at 95°C for 15 seconds and 60°C for 16 minutes, and a final hold at 10°C. Incorporated primers sequences were partially digested using a proprietary method. Ion Torrent Proton adapters were ligated to the amplicons at 22°C for 30 minutes followed by 72°C for 10 minutes, and the library was purified with Agencourt Ampure XT beads (Agencourt Bioscience, Beverly, MA). Libraries were quantified by quantitative polymerase chain reaction (PCR) and 7pM were used for sequencing on an Ion Torrent Proton Sequencer using a PI chip V2 following the manufacturer's protocol. All data were aligned to the hg19/GRCh37 reference genome and were quality trimmed via Ion Torrent Suite version 4.2 (Life Technologies).

Next-Generation Sequencing Data Analysis. SNP and Variation Suite version 8.1 (Golden Helix, Bozeman, MT) and VarSeq Version 1.1 (Golden Helix) were used. After importing the variant call files of each member of the family trio (patient and parents), the variants were organized by pedigree. Using the 1000 genomes Variant Frequencies (phase 1), the Exome Aggregation Consortium Variant Frequency database version 0.3 (Cambridge, MA), and the NHLBI Exome Sequencing Project (https://esp.gs.washington.edu/drupal/) V2 Exome Variant Frequencies, rare variants (minor allele frequency <1%) were filtered. Variants were then classified according to whether they were deemed to be coding. Nonsynonymous and unclassified variants were then scored using the database for nonsynonymous functional predictions (dbNSFP 2.8),[6,7] filtering out variants found to have no damaging score (Polyphen2, SIFT, MutationTaster, MutationAssessor, FATHMM). As well, dbNSFP scores variants with conservation scores (PhyloP and GERP++).

Sanger Sequencing Validation. Sanger sequencing was performed in the patient and his parents to validate the mutation identified by WES (c.1072C>T; p.Arg358*). The following primers were used to sequence exons 2 and 3: forward AGCAAGTGTGAGATCAGCCT, and reverse GGCCAACATAGTGAAACCCC.

Constructs

The constructs generated are summarized here and in Table 1. All PLVAP constructs were cloned into the EcoRI

Table 1. Plasmalemma Vesicle Associated Protein (PLVAP) Constructs Generated and Expected Characteristics

PLVAP Construct	Start PLVAP aa #	End PLVAP aa #	Protein Length (aa)	Protein Molecular Weight (kDa)	Protein + N-Glycosylation (10 kDa)	3xHA (kDa)	Monomer (kDa)	Dimer (kDa)
FL-3xHA	1	442	442	50.59	60.59	3.50	64.09	128.18
389-3xHA	1	389	389	44.91	54.91	3.50	58.41	116.82
357-3xHA	1	357	357	41.16	51.16	3.50	54.66	109.32
R358*	1	357	357	41.16	51.16		51.16	102.32
348-3xHA	1	348	348	40.00	50.00	3.50	53.50	107.00
307-3xHA	1	307	307	35.48	45.48	3.50	48.98	97.96
266-3xHA	1	266	266	30.78	40.78	3.50	44.28	88.56
225-3xHA	1	225	225	25.00	35.00	3.50	38.50	77.00