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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Defects in Nicotinamide-adenine Dinucleotide Phosphate Oxidase Genes *NOX1* and *DUOX2* in Very Early Onset Inflammatory Bowel Disease

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SUMMARY

NOX1 and DUOX2 are the predominant source of intestinal epithelial ROS. Here we identify novel *NOX1* and *DUOX2* variants associated with VEOIBD that result in reduced ROS production, Paneth cell metaplasia and defective host resistance to *C. jejuni*.

BACKGROUND & AIMS: Defects in intestinal innate defense systems predispose patients to inflammatory bowel disease (IBD). Reactive oxygen species (ROS) generated by nicotinamide-adenine dinucleotide phosphate (NADPH) oxidases in the mucosal barrier maintain gut homeostasis and defend against pathogenic attack. We hypothesized that molecular genetic defects in intestinal NADPH oxidases might be present in children with IBD.

METHODS: After targeted exome sequencing of epithelial NADPH oxidases *NOX1* and *DUOX2* on 59 children with very early onset inflammatory bowel disease (VEOIBD), the identified mutations were validated using Sanger Sequencing. A structural analysis of *NOX1* and *DUOX2* variants was performed by homology in silico modeling. The functional characterization included ROS generation in model cell lines and in in vivo transduced murine crypts, protein expression, intracellular localization, and cell-based infection studies with the enteric pathogens *Campylobacter jejuni* and enteropathogenic *Escherichia coli*.

RESULTS: We identified missense mutations in *NOX1* (c.988G>A, p.Pro330Ser; c.967G>A, p.Asp360Asn) and *DUOX2* (c.4474G>A, p.Arg1211Cys; c.3631C>T, p.Arg1492Cys) in 5 of 209 VEOIBD patients. The NOX1 p.Asp360Asn variant was replicated in a male Ashkenazi Jewish ulcerative colitis cohort. Patients with both NOX1 and DUOX2 variants showed abnormal Paneth cell metaplasia. All *NOX1* and *DUOX2* variants showed reduced ROS production compared with wild-type enzymes. Despite appropriate cellular localization and comparable pathogen-stimulated translocation of altered oxidases, cells harboring *NOX1* or *DUOX2* variants had defective host resistance to infection with *C. jejuni.*

CONCLUSIONS: This study identifies the first inactivating missense variants in *NOX1* and *DUOX2* associated with VEOIBD. Defective ROS production from intestinal epithelial cells constitutes a risk factor for developing VEOIBD. *(Cell Mol*

*Authors contributed equally to the study; [§]Participant in the Inter-National Early Onset Pediatric IBD Cohort Study (www.NEOPICS.org).

Abbreviations used in this paper: **AJ, Ashkenazi Jewish; CGD, chronic granulomatous disease; *DUOX2*, dual oxidase 2; HA, human influenza hemagglutinin; IBD, inflammatory bowel disease; FAD, flavin adenine nucleotide; MAF, minor allele frequency; NADPH, nicotinamide-adenine dinucleotide phosphate; *NOX1*, NADPH oxidase 1; PAS, periodic acid–Schiff; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; SNP, single-nucleotide polymorphism; UC, ulcerative colitis; VEOIBD, very early onset inflammatory bowel disease; WT, wild type.**

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Keywords: Inflammatory Bowel Disease; NADPH Oxidase; *NOX1*; *DUOX2*; Reactive Oxygen Species; VEOIBD.

Inflammatory bowel disease (IBD), a complex disease associated with genetic predisposition and environmental factors, is characterized by recurrent intestinal inflammation and microbial dysbiosis. Genomewide association studies link adult IBD to alterations in genes involved in host-microbe interactions.[1,2] Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-generated reactive oxygen species (ROS) are intrinsic to the antimicrobial host defense system of professional phagocytes. Defective ROS production in patients with chronic granulomatous disease (CGD), a rare genetic disorder caused by inactivating alterations of genes required for formation of the penultimate phagocyte oxidase complex (*CYBB*, *CYBA*, *NCF1*, *NCF2*, *NCF4*), confers susceptibility to life-threatening bacterial and fungal infections.[3] Up to 40% of CGD patients develop inflammatory colitis that mimics Crohn's disease.[4] Genetic variants in *NCF4* and *NCF2* that lead to partial attenuation in phagocyte oxidase (NADPH oxidase 2, NOX2) function without causing CGD have been associated with adult and very early onset IBD (VEOIBD).[5,6] We have recently shown that single-nucleotide polymorphisms (SNPs) and rare hypomorphic variants in all components of the NOX2 NADPH oxidase complex are associated with VEOIBD.[7]

A role for ROS production by intestinal epithelial cells in mucosal barrier function and intestinal homeostasis is just emerging.[8] The predominant sources of ROS in the lining of the gastrointestinal tract are the NADPH oxidases NOX1 (NADPH oxidase 1) and DUOX2 (dual oxidase 2), with NOX1 expression restricted mainly to colon, caecum, and ileum, whereas DUOX2 can be found in all segments of the gut.[9] NOX1 and DUOX2 are the catalytic subunits of multimeric, membrane-bound enzymes that generate upon stimulation superoxide and hydrogen peroxide by transfer of electrons from NADPH to molecular oxygen. We[10] and others[11–13] have reported NOX1/DUOX2-mediated ROS production in the intestine and its effect on bacterial pathogenicity and barrier integrity. Here, we describe the identification and characterization of missense mutations in *NOX1* (NM_007052.4, location Xq22) and in *DUOX2* (NG_016992, location 15q15.3) in patients diagnosed with VEOIBD.

Materials and Methods

Study Design

All results are presented according to the STrengthening the REporting of Genetic Association Studies (STREGA) guidelines.[14] Fifty-nine IBD patients diagnosed under the age of 6 years were sequenced for *NOX1* and *DUOX2* by targeted exome sequencing using Agilent SureSelect target enrichment and sequencing (Agilent Technologies, Santa Clara, CA) on the Illumina HiSeq 2000/2500 (Illumina, San Diego, CA) with exon primer and sequencing protocols designed by the Beckman Coulter Genomics (beckmangenomics.com; Beckman Coulter, Brea, CA) as described previously elsewhere.[15] Sanger sequencing was used to verify all genetic defects identified using targeted sequencing of the *NOX1* and *DUOX2* genes at the Centre for Applied Genomics (TCAG; http://www.tcag.ca; Hospital for Sick Children, Toronto, ON, Canada).

Single-nucleotide and insertion/deletion (indel) variants identified by targeted exome sequencing and validated by Sanger sequencing were automatically scanned and manually verified. Furthermore, all variants were also validated using Taqman performed by the Centre for Applied Genomics, Hospital for Sick Children.[15,16] Function and minor allele frequency (MAF) were searched for using the National Heart, Lung, and Blood Institute Exome Sequencing Project (ESP) Exome Variant Server (http://evs.gs.washington.edu/EVS/), the National Center for Biotechnology Information dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/), the National Institute of Environmental Health Sciences FuncPred (http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm), Polyphen2 (http://genetics.bwh.harvard.edu/pph2/),[17] SIFT (http://sift.jcvi.org/),[18] FastSNP (http://fastsnp.ibms.sinica.edu.tw/),[19] Human Splicing Finder (http://www.umd.be/HSF/),[20] and pfSNP (http://pfs.nus.edu.sg/).[21]

Setting

Patients included in the study were recruited from the Inflammatory Bowel Disease Clinic at the Hospital from Sick Children, University of Toronto. They were diagnosed with VEOIBD between the years 1994 and 2012 and had a confirmed diagnosis of IBD before the age of 6 years. Although there is no consensus on the definition of VEOIBD, we have used the stricter definition based on our recent modification (diagnosis <6 years of age)[5,22,23] of the Paris classification.[24] Our definition, which is more stringent and includes more severe cases that are more likely to cause monogenic forms of the disease, has been used to identify risk variants in this age group. There were no exclusion criteria for patients diagnosed with VEOIBD; however, patients with a known immunodeficiency or a clinical diagnosis of CGD were excluded because these patients were not defined as VEOIBD. The five identified patients were screened and were found negative for pathogenic mutations in *IL10RA*, *IL10RB*, *IL10*, *XIAP*, *TTC7A*, as well as genes involved in CGD (*RAC1/2*, *NCF1/2/4*, and *CYBB, CYBA*)[23,25] and *NOD2* and *ATG16L1* variants associated with IBD.

Participants

This was a cohort study that examined the genetics of VEOIBD patients. Fifty-five VEOIBD patients were recruited from the Hospital for Sick Children, Toronto, Canada. A second cohort of VEOIBD patients was recruited through NEOPICS (www.NEOPICS.org). The replication cohort comprised 1477 Crohn's disease cases, 559 ulcerative colitis cases, and 2614 healthy controls, all with genetically verified Ashkenazi Jewish ancestry by principal components analysis.