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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Environmental Enteric Dysfunction Includes a Broad Spectrum of Inflammatory Responses and Epithelial Repair Processes

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SUMMARY

The host transcriptome in feces was characterized in 259 rural Malawian children at risk for environmental enteric dysfunction. A broad range of immune activation and defects in cell adhesion were found, coupled with decreased mucin expression, elucidating the pathobiology of this condition.

BACKGROUND & AIMS: Environmental enteric dysfunction (EED), a chronic diffuse inflammation of the small intestine, is associated with stunting in children in the developing world. The pathobiology of EED is poorly understood because of the lack of a method to elucidate the host response. This study tested a novel microarray method to overcome limitation of RNA sequencing to interrogate the host transcriptome in feces in Malawian children with EED.

METHODS: In 259 children, EED was measured by lactulose permeability (%L). After isolating low copy numbers of host messenger RNA, the transcriptome was reliably and reproducibly profiled, validated by polymerase chain reaction. Messenger RNA copy number then was correlated with %L and differential expression in EED. The transcripts identified were mapped to biological pathways and processes. The children studied had a range of %L values, consistent with a spectrum of EED from none to severe.

RESULTS: We identified 12 transcripts associated with the severity of EED, including chemokines that stimulate T-cell proliferation, Fc fragments of multiple immunoglobulin families, interferon-induced proteins, activators of neutrophils and B cells, and mediators that dampen cellular responses to hormones. EED-associated transcripts mapped to pathways related to cell adhesion, and responses to a broad spectrum of viral, bacterial, and parasitic microbes. Several mucins, regulatory factors, and protein kinases associated with the maintenance of the mucous layer were expressed less in children with EED than in normal children.

CONCLUSIONS: EED represents the activation of diverse elements of the immune system and is associated with widespread intestinal barrier disruption. Differentially expressed transcripts, appropriately enumerated, should be explored as potential biomarkers. *(Cell Mol Gastroenterol Hepatol 2016;2:158–174; http://dx.doi.org/10.1016/j.jcmgh.2015.12.002)*

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Stunting, defined as a height-for-age z score (HAZ) of less than -2, affects 26% of all children younger than the age of 5 years worldwide.[1,2] Stunting is associated with reduced neurocognitive capability, diminished immunocompetence, 20% of disability-adjusted life years lost in this age group, and more than 2.1 million deaths annually.[2]

Optimal gut health encompasses effective dietary nutrient absorption and a mucosal immune response that confines microbes to the lumen without inducing chronic tissue inflammation. Environmental enteric dysfunction (EED) is an asymptomatic, diffuse villous atrophy of the small bowel associated with chronic mucosal T-cell infiltration and reduced paracellular integrity.[3] EED is highly prevalent, often without gastrointestinal symptoms, in poor children in the developing world.[4,5]

EED typically is assessed with a dual sugar permeability test, whereby mannitol (molecular weight, 182 daltons) and lactulose (molecular weight, 342 daltons) are ingested under controlled conditions and quantified in the urine. Both sugars are neither degraded in the upper gastrointestinal tract nor systemically metabolized after absorption, and are excreted rapidly in the urine.[6] Lactulose is a disaccharide, which can be absorbed only by passively crossing disrupted cell junctions, and thus the amount of this sugar in the urine reflects small-bowel permeability.[6,7] Mannitol, a monosaccharide, is absorbed across cell membranes and between

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Abbreviations used in this paper: dHAZ, change in height-for-age z score; EED, environmental enteric dysfunction; FARMS, factor analyses for robust microarray summarization; G-CSF, granulocyte colony–stimulating factor; HAZ, height-for-age z score; IRON, iterative rank order normalization; KEGG, Kyoto Encyclopedia of Genes and Genomes; mRNA, messenger RNA; %L, lactulose permeability; qPCR, quantitative polymerase chain reaction; RMA, robust multi-array average.

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cell junctions and is included to normalize lactulose uptake and excretion to the mucosal surface area, and to control for variations in gastric emptying time. Both the ratio of urinary lactulose to mannitol and the fraction of lactulose that is excreted in the urine (lactulose permeability [%L]) have been used to assess gut health. The dual sugar assay, although imperfect, is a theoretically sound measurement test of gut health.[7]

Much has been learned about health and disease in the past decade by using agnostic surveys of the human transcriptome,[8] including, in recent years, reliance on RNA deep sequencing to profile transcriptional response to injury. Unfortunately, these methods have required RNA samples larger than 1 μg that have been processed to remove inhibitors of nucleic acid hybridization and nonhuman RNA. This requirement has limited our understanding of the host transcriptome analyses of feces from individuals.

This report details the development and application to a human cohort of a novel RNA selective isolation procedure from human feces, coupled with high-density, whole human transcriptome microarray technology to interrogate samples from 259 rural Malawian children with varying states of EED.

Methods

Study Design

This was a prospective cohort observational study of rural African children at high risk for EED. The primary outcomes were the correlation between %L and expression levels of protein coding genes, based on data that %L correlates with linear growth in this population.[9,10] Secondary outcomes were associations with Kyoto Encyclopedia of Genes and Genomes (KEGG) and canonical pathways in EED.

Eligible Subjects

The study was conducted in rural Malawi, where populations practice subsistence farming (corn and beans), and reside in mud and thatch homes. Water is collected from boreholes and wells; electricity is unavailable. Inclusion criteria consisted of subjects between 12 and 61 months of age who reside in 1 of 6 rural communities under research surveillance, and included 810 children in total.[11–13] This included a spectrum of children with EED, from no EED to severe EED. Children were excluded if they had a chronic disability or disease, severe acute malnutrition, or were receiving therapy for tuberculosis. All subjects were interviewed and examined by a physician and found to be free of pathologic conditions. Weight, length, and mid-upper-arm circumferences were measured by trained and monitored staff to determine nutritional status.

Dual Sugar Absorption Testing

Dual sugar permeability testing was conducted in a supervised setting, and complete consumption of the sugars and collection of all urine during the subsequent 6 hours was verified.[12] Children consumed no food or drink for 8 hours before drinking 20 mL of water into which 1 g of mannitol and 5 g of lactulose were dissolved. This was administered immediately after children voided. A dual sugar permeability test was considered successfully completed when all urine was collected for at least 4 hours after ingestion of the sugars, without spillage of dosing sugars or urine. Urine volumes were measured using a graduated cylinder, and a 2-mL aliquot was flash-frozen and shipped to the Baylor College of Medicine (Houston, TX) where urinary lactulose was measured using high-pressure liquid chromatography.[14,15] EED severity was assigned using population data from a larger clinical study such that the children with %L less than 0.2 were designated as not having EED, and those with %L greater than 0.2 and less than 0.7 were designated as having intermediate EED, and those with %L greater than 0.7 were designated as having severe EED.[9] The transformation $\log_2$ (%L*100) was used to determine linear correlations between %L and microarray data.

Stool Collection

Fresh stools were collected before the dual sugar absorption testing using a small, clean, nonabsorbent, plastic diaper. The stools were transferred immediately to cryovials and flash-frozen in liquid nitrogen. Samples were transferred to a -80°C freezer and transported to Washington University (St. Louis, MO), where they then were processed and analyzed for the human fecal transcriptome as outlined in Figure 1 and detailed later.

Samples Chosen for Transcriptome Analyses

We chose 259 children for whole-transcriptome analysis on the basis of a mannitol excretion greater than 3%, a total urine volume greater than 15 mL, and a broad distribution of urinary %L values, including normal children. The mannitol was used as a test validation criterion because very small amounts of mannitol absorption indicate very rapid transit intestinal transit times, which distorts the validity of %L as a measure of gut integrity.

Enriching Fecal Samples for Exfoliated Enterocytes by Differential Centrifugation

Fecal samples were enriched for human cells by differential centrifugation before RNA extraction. Approximately 300–500 mg of frozen stool was transferred to a 15-mL conical tube with 10–15 zirconium/silica beads (2.3 mm) and 3 mL of Hank's balanced salt solution (Gibco/Life Technologies, Grand Island, NY) with 0.05% Tween-20 (Sigma, St Louis, MO). The samples were vortexed gently for 5 minutes to suspend aggregates. The buffer volume was increased to 10 mL and incubated at 4°C on a rotator for 10 minutes, followed by centrifugation at 1000 rpm (500*g*) for 10 minutes. The supernatant was removed and the pellet was resuspended in 10 mL of Hank's balanced salt solution/Tween-20 buffer and incubated and centrifuged as before.

Extracting and Assessing Enriched Fecal RNA

Total fecal nucleic acids were extracted from human-enriched pellets and bacterial-enriched supernatants