Gastroenterology 2015;148:533–536

BRIEF REPORT

Truncating Mutation in the Nitric Oxide Synthase 1 Gene Is Associated With Infantile Achalasia

Eyal Shteyer,[1,*] **Simon Edvardson**,[2,*] **Sarah L. Wynia-Smith**,[3,*] **Ciro Leonardo Pierri**,[4,*] **Tzili Zangen**,[5,*] Saar Hashavya,[6] Michal Begin,[2] Barak Yaacov,[7] Yuval Cinamon,[7] Benjamin Z. Koplewitz,[8] Amos Vromen,[9] Orly Elpeleg,[7] and Brian C. Smith[3]

[1]Pediatric Gastroenterology Unit, Department of Pediatrics; [2]Neuropediatric Unit, Hadassah-Hebrew University Medical Center, Jerusalem, Israel; [3]Department of Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin; [4]Department of Biosciences, Biotechnologies and Biopharmaceutics, University of Bari, Bari, Italy; [5]Pediatric Gastroenterology Unit, E Wolfson Medical Center, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel; [6]Emergency Medicine Department of Pediatrics; [7]Monique and Jacques Roboh Department of Genetic Research, Hadassah-Hebrew University Medical Center, Jerusalem, Israel; [8]Department of Medical Imaging; and [9]Department of Pediatric Surgery, Hadassah-Hebrew University Medical Center, Jerusalem, Israel

See Covering the Cover synopsis on page 459.

Nitric oxide is thought to have a role in the pathogenesis of achalasia. We performed a genetic analysis of 2 siblings with infant-onset achalasia. Exome analysis revealed that they were homozygous for a premature stop codon in the gene encoding nitric oxide synthase 1. Kinetic analyses and molecular modeling showed that the truncated protein product has defects in folding, nitric oxide production, and binding of cofactors. Heller myotomy had no effect in these patients, but sildenafil therapy increased their ability to drink. The finding recapitulates the previously reported phenotype of nitric oxide synthase 1–deficient mice, which have achalasia. Nitric oxide signaling appears to be involved in the pathogenesis of achalasia in humans.

Keywords: Human Genetics; Esophageal Disorder; Swallow; Muscle Relaxation.

Achalasia is a rare primary esophageal motility disorder of unknown etiology. It can present as an isolated finding or as part of a syndrome, including Down syndrome, Allgrove (Achalasia-addisonianism-alacrimia) syndrome, familial visceral neuropathy, and achalasia-microcephaly syndrome.[1] Several achalasia mouse models are known, resulting from mutations in the Rassf1a, nitric oxide synthase 1, Kit, or Spry2 genes.[1] However, mutation and common polymorphism analysis of these genes in patients with achalasia yielded negative results.[1]

The subjects of this report are 2 siblings: a 6-year-old girl (II-1) and a 2.5-year-old boy (II-3) (Figure 1*A*), the first and third children to first-cousin parents of Arab origin. The parents and their second child were healthy and the extended family history was noncontributory. The family first sought medical advice for patient II-1 when she was 5 months old because of recurrent vomiting since birth, dysphagia, and failure to thrive. Upper gastrointestinal series and high-resolution esophageal manometry were compatible with the diagnosis of type III (spastic) achalasia (Figure 1*D* and *E*). Heller myotomy was performed at 3 years, however, there was no improvement in her ability to swallow, and she remained fed through a gastrostomy tube. Patient II-3 was diagnosed at the age of 2 months with type III (spastic) achalasia. Both children were diagnosed with autism (see Supplementary Material).

Because of the parental consanguinity, we suspected a founder mutation transmitted in an autosomal-recessive manner. Exome analysis in patient II-1 DNA (detailed in the Supplementary Material) disclosed a homozygous premature termination codon in the nitric oxide synthase 1 (NOS1) gene at residue Tyr1202 instead of the normally occurring termination codon at residue 1435 (Figure 1*B* and *C*). The encoded neuronal nitric oxide synthase (nNOS) protein catalyzes the oxidation of L-arginine and nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) to L-citrulline, $NADP^+$, and NO. Tyr1202 resides in the C-terminal electron-supplying reductase module (NOSred) that binds the flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) cofactors as well as the NADPH substrate (Figure 2*A*). We hypothesized that the Tyr1202Ter (Y1202X) mutation abolishes proper NOS cofactor assembly and therefore NO synthesis (for details see Supplementary Material).

Assessment of NOS1 messenger RNA in fibroblasts disclosed very low levels of the normal transcript in both affected and unaffected individuals (see Supplementary Material). Because NOS1-expressing tissue was not available for additional studies, we measured the rates of NO synthesis and NADPH oxidation for purified wild-type (WT) and Y1197X (equivalent to Y1202X in human nNOS;

*Authors share co-first authorship.

Abbreviations used in this paper: **FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS1, nitric oxide synthase 1; NOSred, nitric oxide synthase c-terminal electron-supplying reductase module; WT, wild-type.**

0016-5085/$36.00
http://dx.doi.org/10.1053/j.gastro.2014.11.044

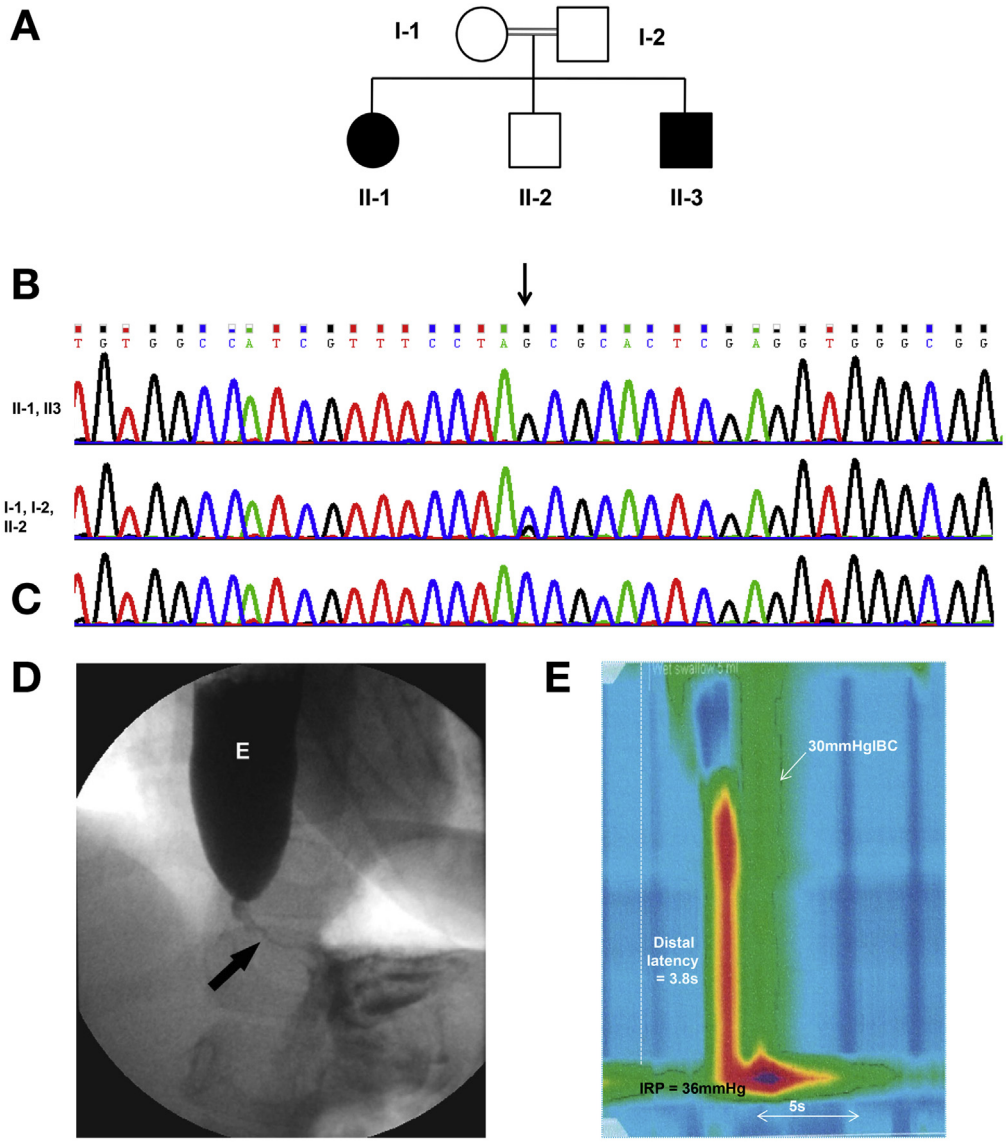

Figure 1. (*A*) Family pedigree, patients are designated by *filled symbols* and the mutation genotype is indicated (*B*). NOS1 genomic sequence around the mutation site in the DNA samples of the family members, and of a normal control (*C*). An upper gastrointestinal series (*D*) and a high-resolution manometry (*E*) of patient II-1. There is severe narrowing (*arrow*) of the distal esophagus (Eso). High-resolution manometry (*E*) after 5-mL water swallow shows premature spastic contraction (distal latency 3.8 s) and impaired lower esophageal sphincter relaxation (integrated relaxation pressure [IRP] of 36 mmHg), exhibited in 6 of 10 swallows, compatible with type III achalasia.

Figure 2*B*) nNOS from *Rattus norvegicus*. Both nNOS WT (160 kDa) and Y1197X (135 kDa) migrated at their expected molecular weights (Figure 2*C*). As predicted, NO formation was not detectable for rat nNOS Y1197X. WT rat nNOS exhibited a robust steady-state rate of NO formation (0.20 ± 0.07/s) (Figure 2*D*). Similarly, the rate of NADPH oxidation for rat nNOS Y1197X was <5% of the rate observed for WT rat nNOS (0.013 ± 0.002/s vs 0.31 ± 0.04/s) indicating a strong defect in NADPH oxidation for the truncated nNOS (Figure 2*D*).

In order to interpret the loss of NO synthase activity observed for the prematurely truncated nNOS protein, two 3-dimensional comparative models were built (see Supplementary Material) based on the crystal structure of the *R norvegicus* NOSred domain,[2] one for the human WT NOSred domain and the other for the truncated human NOSred domain (Figure 2*E*). Our 3-dimensional model of the human WT NOSred domain contains bound NADPH, FAD, and FMN. In the truncated NOSred model protein residues involved in the FAD and NADPH binding are missing (Figure 2*E*). Therefore, the mutation likely not only adversely affects correct NOS folding, but also NADPH and FAD binding, resulting in in the observed loss of NO synthase activity, which would not be responsive to flavin cofactor supplementation.

NO was previously shown to play a role in gastrointestinal tract nerve–mediated relaxation, exemplified in rat anococcygeus,[3,4] in dog duodenum,[5] and specifically in the relaxation of the circular muscle of the lower esophageal sphincter of opossum.[6] In addition, NOS immunoreactivity is localized specifically in nerve cell bodies of the myenteric plexus and nerve fibers in the circular muscle of the intestine.[7] Accordingly, NO is thought to play a role in achalasia in humans, as low nNOS activity was reported in biopsies from patients with achalasia[8] and reduction of lower esophageal sphincter pressure was reported in achalasia patients treated with Sildenafil, which blocks phosphodiesterase type 5, preventing degradation of the secondary messenger cyclic