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Hirschsprung disease is associated with an L286P mutation in the fifth transmembrane domain of the endothelin-B receptor in the N-ethyl-N-nitrosourea-induced mutant line

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Abstract: Hirschsprung disease (HSCR), or colonic aganglionosis, is a congenital disorder characterized by the absence of intramural ganglia along variable lengths of the colon, resulting in intestinal obstruction. It is the most common cause of congenital intestinal obstruction, with an incidence of 1 in 5,000 live births. N-ethyl-N-nitrosourea (ENU)-induced mutagenesis is a powerful tool for the study of gene function and the generation of human disease models. In the current study, a novel mutant mouse with aganglionic megacolon and coat color spotting was generated by ENU-induced mutagenesis. Histological and acetylcholinesterase (AChE) whole-mount staining analysis showed a lack of ganglion cells in the colon in mutant mice. The mutation was mapped to chromosome 14 between markers *rs30928624* and *D14Mit205* (Chr 14 positions 103723921 bp and 105054651 bp). The *Ednrb* (Chr 14 position 103814625–103844173 bp) was identified as a potential candidate gene in this location. Mutation analysis revealed a T>C missense mutation at nucleotide 857 of the cDNA encoding endothelin receptor B (EDNRB) in which a proline was substituted for the highly conserved Lys-286 residue (L286P) in the fifth transmembrane (TM V) domain of this G protein-coupled receptor. The mutant mouse was named *Ednrb^{m1yzcm}* (*Ednrb*; mutation 1, Yangzhou University Comparative Medicine Center). The results of the present study implicate the structural importance of the TM V domain in *Ednrb* function, and the *Ednrb^{m1yzcm}* mouse represents a valuable model for the study of HSCR in humans.

Key words: *Ednrb*, ENU, Hirschsprung disease (HSCR), mouse

Introduction

Hirschsprung disease (HSCR), or colonic aganglionosis, is a congenital malformation characterized by the absence of intramural ganglia along variable lengths of the colon, resulting in intestinal obstruction with severe constipation in infants and adults. It is the most common

cause of congenital intestinal obstruction, with an incidence of 1 in 5,000 live births, and it occurs more frequently in males than in females (4:1). HSCR has been divided into short-segment HSCR (S-HSCR, 80%), long-segment HSCR (L-HSCR, 15%), and total colonic aganglionosis (TCA, 5%) based on the length of the aganglionic tract [2, 8, 9].

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Several HSCR-susceptibility genes have been identified, including the *RET* proto-oncogene [21], the endothelin receptor B gene (*EDNRB*) [1, 4, 5, 14], the endothelin-3 gene (*EDN3*) [7, 10, 12], *GDNF* [3, 13, 16], and the *SOX10* gene [22].

EDNRB belongs to the superfamily of rhodopsin-like G protein-coupled receptors (GPCRs), which consist of a long extracellular N-terminus sequence, seven helical transmembrane domains (TMDs), 3 extracellular and 3 intracellular loops, and a cytoplasmic C-terminus tail. The receptor recognizes a family of small peptides known as endothelins [17, 18]. Mutations in the *Ednrb* gene have been linked to Hirschsprung disease in mice and humans [1, 4, 5, 14].

N-ethyl-N-nitrosourea (ENU)-induced mutagenesis is a powerful tool for the study of gene function and the generation of human disease models. In this paper, a new missense mutation in *Ednrb* resulted in an HSCR phenotype. This new mutant was generated in a phenotype-driven screen of mice that had been mutagenized with ENU, and it is described here along with the phenotypic characterization of the mutant mice. Mutation analysis revealed a T>C missense mutation in *Ednrb* exon 4 in which the highly conserved Lys-286 residue in the fifth transmembrane helix of the EDNRB was substituted with a proline (L286P). The mutant mouse was named *Ednrb^{myzcm}* (*Ednrb*; mutation 1, Yangzhou University Comparative Medicine Center).

Materials and Methods

Mice

C57BL/6J (B6) and DBA/2J (D2) mice were obtained from the Shanghai Laboratory Animal Center (Shanghai, China). This study was conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Research Council. The Animal Care and Use Committee of Yangzhou University approved all animal experiments and procedures (Approval ID: SYXK [Su] 2012–0029).

The *Ednrb^{myzcm}* mouse was generated via ENU mutagenesis using B6 mice. The *Ednrb^{myzcm}* heterozygotes were mated to B6 females to confirm inheritance test results. Heterozygous mutants were intercrossed to generate homozygous mutants.

Histological and acetylcholinesterase (AChE) whole-mount staining analysis

For Harris hematoxylin and eosin-Y (H&E) staining, colon tissues were dissected and fixed in 4% paraformaldehyde in phosphate-buffered saline, dehydrated, embedded in wax, sectioned at a thickness of 6 μ m, and then stained with H&E.

For AChE whole-mount staining, the guts from pups at postnatal day 14 were dissected as a single piece from the proximal esophagus to the distal colon. The mesenteric attachments and pancreas were removed, and the guts were fixed in 4% paraformaldehyde for 1–2 h at 4°C. The tissues were then transferred to saturated sodium sulfate and stored overnight at 4°C. The tissues were then incubated in buffer (ethopropazine HCl [Sigma] 0.2 mM, acetylthiocholine iodide [Sigma] 4 mM, glycine 10 mM, cupric sulfate 2 mM, and sodium acetate 65 mM [pH=5.5]) for 2–4 h. Staining for AChE was done by incubation for 1.5 min in sodium sulfide (1.25%, pH=6). Tissue was rinsed extensively with water before photographing under a dissecting microscope.

Mutation mapping and identification

Ednrb^{myzcm} heterozygotes from B6 mice were mated to D2 mice to generate F₁ mice. The F₁ mice were then intercrossed to generate F₂ mice. DNA samples of F₂ homozygous mutants were prepared from tail samples by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. PCR was used to screen DNA samples for microsatellite markers. PCR products were separated on 4% agarose gels by electrophoresis and analyzed.

Total RNA was isolated from the heads of postnatal day 10 mice using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using a RevertAid First-Strand cDNA Synthesis Kit (Thermo Scientific Fermentas, St. Leon-Rot, Germany) with oligo (dT) 18 primers. RT-PCR for *Ednrb* was performed with the following primers: forward 5'-TTGGCTGGGGTAGCT-GACTTAA-3' and reverse 5'-CACACCTTTCTGCTAG-CATGGTTT-3'. The PCR conditions consisted of one cycle of denaturation for 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 61°C, and 1.5 min at 72°C; and finally one cycle of elongation for 5 min at 72°C. PCR products were then purified and sequenced.

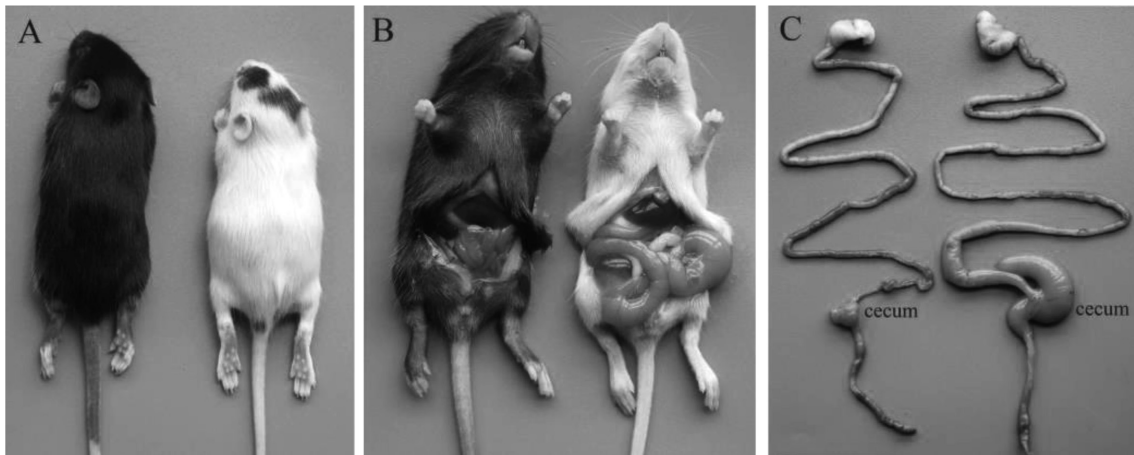


Fig. 1. Phenotype of *Ednrb^{mlyzcm}* mice. (A) Note the white coat color of a homozygous *Ednrb^{mlyzcm}* mouse (right). (B) Autopsy of the wild type (left) and homozygous *Ednrb^{mlyzcm}* (right) mice. (C) Dissection of the entire gastrointestinal tract from homozygous *Ednrb^{mlyzcm}* (right) mice revealed a distended colon, cecum, and ileum.

Results

Mutant mouse phenotype

The founder of the *Ednrb^{mlyzcm/+}* mutants was the progeny of an ENU-treated B6 male mouse and had a cluster of white spots on the abdomen. The mutant mouse was mated with wild-type B6 mice, and 9/25 of the progenies showed a similar phenotype. Homozygous mutants, generated by intercross of the heterozygous mutants, showed extensive white spotting of the coat. Well-demarcated, normally pigmented patches of coat were seen most often in the head and hip regions (Fig. 1A). The shapes of these pigmented patches were irregular and differed randomly from mouse to mouse. The eyes were dark in all cases. These mice appeared healthy during the first week after birth but had increasing abdominal distention after 1 week of age (Figs. 1B and 1C). Ultimately, all homozygotes died before postnatal 21 day.

Lack of ganglion cells in the colon in Ednrb^{mlyzcm} homozygous mice

Histological examination of longitudinal sections of the distal portion of the colon from *Ednrb^{mlyzcm}* homozygous mice revealed a lack of myenteric (Auerbach) ganglia (Figs. 2A and 2B).

To more readily visualize the absence of ganglion cells in the colon, the bowels of postnatal day 14 mice were examined using whole-mount AChE histochemistry in order to stain cell bodies and nerve fibers. AChE staining of colons from wild-type mice demonstrated a rich

plexus of interconnected ganglion cells. However, in *Ednrb^{mlyzcm}* homozygous mice, no ganglion cells were visible, although large nerve fibers, extending proximally from the distal colon, were apparent (Figs. 2C and 2D).

A mutation in the Ednrb gene of Ednrb^{mlyzcm} mice

For initial mapping, we tested genomic DNA from 24 F₂ homozygous mutants with microsatellite markers across the whole genome. We mapped the mutation to chromosome 14 and found no significant linkages with other chromosomal loci. For fine mapping, 191 F₂ homozygous mutants were tested, and the mutation was determined to be between markers *rs30928624* and *D14Mit205* (Chr 14 positions 103723921 bp and 105054651 bp) (Fig. 3). A total of 3 protein coding genes are localized within this interval. We prioritized candidate genes on the basis of expression patterns, likely function, and known human or mouse mutant phenotypes. A Hirschsprung-associated gene, *Ednrb* (Chr 14 position 103814625–103844173 bp), was thought to be a particularly good candidate to examine closely for mutation.

A single nucleotide change was found in the *Ednrb* gene, a T/C substitution, in exon 4 at nucleotide position 857 of the *Ednrb*-coding region. This substitution converted the Lys-286 residue in the fifth transmembrane helix of the EDNRB to a proline residue (L286P). Sequence alignment across multiple species revealed that this aspartic acid residue is highly conserved among vertebrates (Fig. 4).

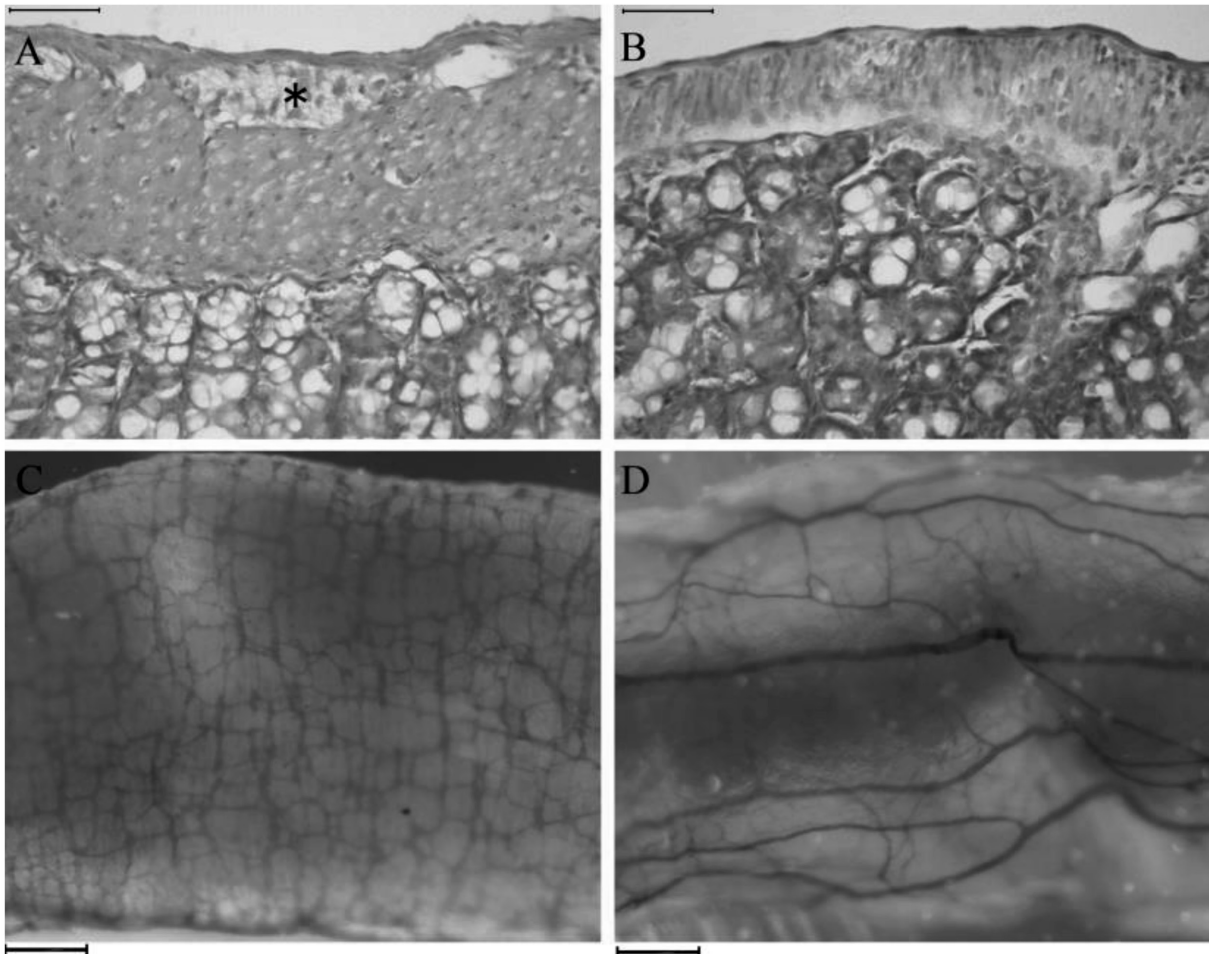


Fig. 2. HE and AChE whole-mount staining of the intestine. (A and B) HE pathological tissue examination confirmed that no ganglia were present in the aganglionosis segment. The upper panel represents H&E staining of the colon from (A) a wild-type mouse and (B) a homozygous *Ednrb^{mlyzcm}* mouse. HE staining of a wild-type colon, demonstrating myenteric (Auerbach) ganglionic cells (indicated by an asterisk), and homozygous *Ednrb^{mlyzcm}* colon, in which ganglionic cells are not present. Scale bar: 50 μm . (C and D) AChE whole-mount staining to visualize the ganglia architecture in the enteric nervous system. (C) Wild-type mouse, (D) *Ednrb^{mlyzcm}* homozygous mouse. A dense plexus of neurons is present in the wild-type colon. Although there are thick nerve fibers in the homozygous *Ednrb^{mlyzcm}* colon, no ganglion cells are present. Scale bar: 200 μm .

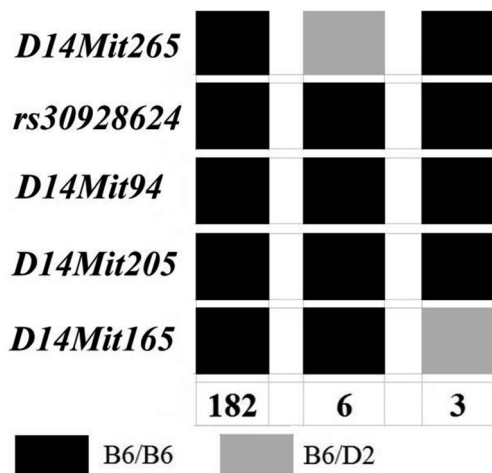


Fig. 3. Genetic mapping places the mutant in the region between markers *rs30928624* and *D14Mit205*. Primary markers used to refine the map position are listed on the left from the centromere (top), and the number of animals in each genotypic class is shown at the bottom.

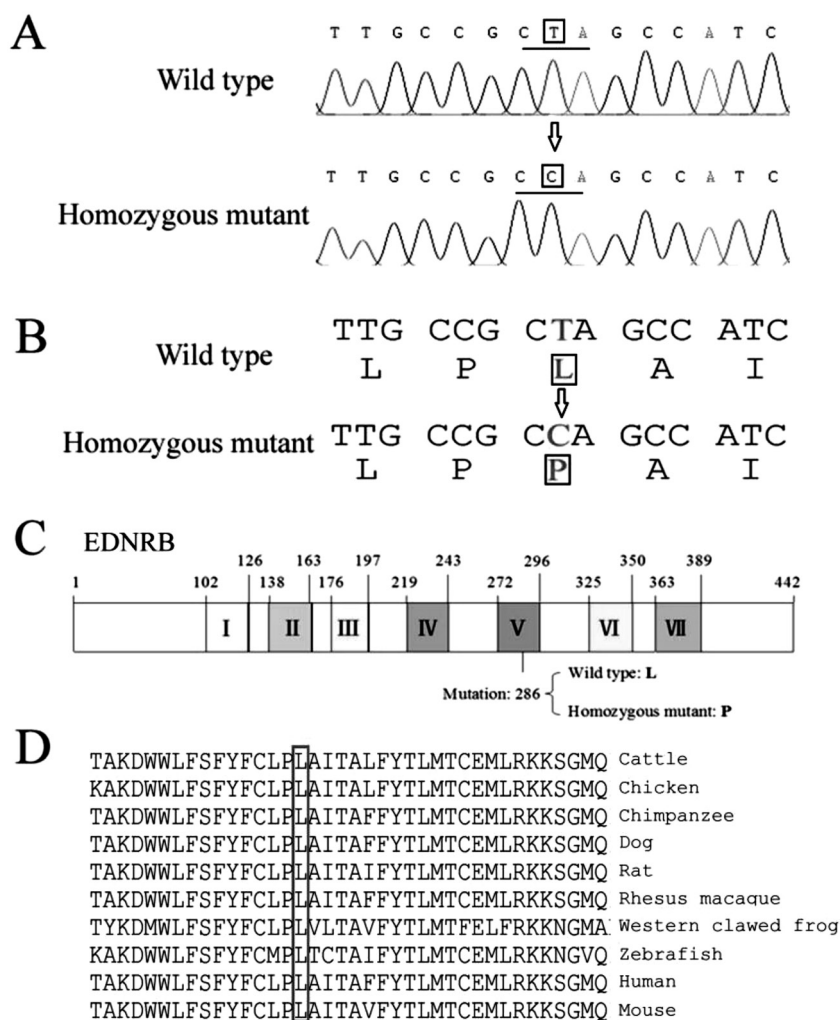


Fig. 4. A point mutation in *Ednrb*^{mlyzcm}. (A) Sequence analysis of the *Ednrb* gene in wild-type B6 and homozygous *Ednrb*^{mlyzcm} mice. (B) Translation of wild-type and homozygous *Ednrb*^{mlyzcm} alleles flanking the mutation site. (C) Diagram of the EDNRB protein structure with the approximate position of the L286P mutation identified in *Ednrb*^{mlyzcm}. The 7 helical transmembrane domains are labeled within the boxes. (D) Sequence alignment of mouse EDNRB with other EDNRB amino acid sequences.

Discussion

The current study presents a novel ENU-induced mutant mouse model, *Ednrb*^{mlyzcm}, with a coat color pigmentation anomaly and megacolon. Histological and AChE examination revealed that the megacolon of the mutant was associated with aganglionosis. The *Ednrb*^{mlyzcm} allele was found to be produced by a T>C transition at nucleotide 857 of the *Ednrb*-coding region, which lead to the substitution of proline for lysine at amino acid residue 286.

Hirschsprung disease is a congenital disease that is

characterized by an aganglionic megacolon with an absence of enteric ganglia and a lack of innervation to the lower gastrointestinal tract. Precursors of myenteric ganglion neurons originate in the vagal neural crest and migrate in a rostral-to-caudal direction along the developing gastrointestinal tract. The most widely accepted etiopathogenetic hypothesis for HSCR is based on a defect of craniocaudal migration of neuroblasts, which originate from the neural crest and travel to the developing gut, followed by cell proliferation and differentiation [11, 19]. Mice with a targeted null disruption of either the *Ednrb* or *Edn3* gene exhibit an identical phenotype,

indicating that the interaction of *Edn3* with *Ednrb* is crucial for normal development of these neural crest-derived tissues [6].

Upon ligand stimulation, the EDNRB receptor activates a number of intracellular signaling mechanisms, including the phospholipase C- β pathway, via heterotrimeric G proteins, leading to an acute increase in cytoplasmic Ca^{2+} concentration [17]. Biochemical studies have demonstrated that different fifth transmembrane (TM V) domains contribute to ligand binding and intracellular signal transduction properties of the receptors [15, 17, 18]. W276C mutations in the TM V domain of EDNRB receptors have been identified in human patients with Hirschsprung disease. Other studies have reported that a W276C mutation affects the intracellular signal response of EDNRB [18]. A P285L mutation that causes aganglionic megacolon and white coat color has also been identified in the TM V domain of the EDNRB receptor in *Ednrb*^{27Pub} mice [20]. Lys-286 is situated in the TM V domain and is highly conserved among vertebrates. The *Ednrb*^{m^{lyzcm} allele harbors an L286P mutation in the TM V domain of the EDNRB receptor that causes aganglionic megacolon and white coat color, suggesting that Lys-286 is important for proper EDNRB function.}

Conflicts of Interest

All authors declare that they have no conflicts of interest regarding this article.

Acknowledgments

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