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Decreased expression of NEDL2 in Hirschsprung's disease

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ABSTRACT

Purpose: NEDD4-like ubiquitin protein ligase 2 (NEDL2) plays an important role in many physiological and pathological processes. NEDL2 is a positive regulator of GDNF/Ret signaling during enteric neurogenesis. Mice lacking NEDL2 exhibit decreased numbers of enteric neurons, progressive bowel dysmotility and intestinal hypoganglionosis. We designed this study to investigate the expression of NEDL2 in the normal human colon and in HSCR.

Methods: HSCR tissue specimens (n = 10) were collected at the time of pull-through surgery and divided into aganglionic and ganglionic segments. Colonic control samples (n = 10) were obtained from patients with imperforate anus at the time of colostomy closure. Immunolabeling of NEDL2 was visualized using confocal microscopy to assess protein distribution, while Western blot analysis was undertaken to quantify NEDL2 protein expression.

Results: Confocal microscopy revealed that NEDL2-immunoreactivity colocalized with ICCs and neurons within the submucosa, myenteric plexus and smooth muscle in controls and ganglionic specimens, with markedly reduced NEDL2-immunoreactivity in aganglionic specimens. Western blotting revealed high levels of the NEDL2 protein in normal controls and the ganglionic region of HSCR, while there was a marked decrease in NEDL2 protein expression in the aganglionic region of HSCR.

Conclusion: We report, for the first time, the expression of NEDL2 in the human colon. The decreased expression of NEDL2 in the aganglionic colon suggests that NEDL2 may play a role in the pathophysiology of HSCR.

The enteric nervous system (ENS) is a network of neurons and glia that lies within the walls of the gastrointestinal tract. This network is divided into two main plexuses, the submucosal plexus and the myenteric plexus, made up of ganglia containing various subtypes of neurons [1]. These neurons express neurotransmitters, as a way of communicating with each other and with neighboring interstitial cells of Cajal (ICCs), platelet-derived growth factor receptor alpha-positive (PDGFRα$^+$) cells and smooth muscle cells (SMCs). Together, these cells regulate intestinal secretory activities and persistalsis. ICCs are known as the pacemaker cells of the gut, and are responsible for the generation of slow waves. PDGFRα$^+$ cells are a recently documented type of interstitial cell found to be involved in purinergic neurotransmission within the human colon [1]. SMCs are responsible for smooth muscle contraction and relaxation.

Ubiquitination is a post-translational protein modification that is critical for a number of cellular processes. Ubiquitination involves the covalent attachment of the 8 kDa protein ubiquitin to one or more lysine residues in the substrate protein to signal proteins for degradation, altered localization, trafficking or function [2][3]. Ubiquitination occurs via a three step process involving an E1 ubiquitin activating enzyme, an E2 ubiquitin conjugating enzyme and an E3 ubiquitin protein ligase [2].

The mammalian Nedd4 family of E3 ubiquitin ligases is a family of proteins comprising nine members: Nedd4-1, Nedd4-2, Smurf1, Smurf2, WWP1, WWP2, Itch, NEDL1 and NEDL2 [4]. NEDL2 has been reported to be a positive regulator of glial cell line-derived neurotrophic factor (GDNF) GDNF/Ret signaling, which plays a crucial role during the proliferation, migration and differentiation of neural crest cells. A recent study has reported that mice lacking the NEDL2 ubiquitin ligase exhibit decreased numbers of enteric neurons, progressive bowel motility defects and intestinal hypoganglionosis [5]. We hypothesized that NEDL2 expression is decreased in HSCR; therefore we designed this study to investigate the expression of the NEDL2 protein in the normal human colon and in HSCR bowel.

1. Materials and methods

1.1. Tissue samples

This study was approved by the Ethics Medical Research Committee, Our Lady's Children's Hospital Crumlin, Dublin, Ireland (Ref. GEN/292/12) and tissue samples were obtained with informed parental consent. HSCR specimens from 10 patients who underwent pull-through surgery were studied. Patients were aged 6 ± 3 months old. No additional health issues existed in these patients. Colonic control samples included 10 specimens from patients who underwent colostomy closure following surgical correction of imperforate anus. Control samples were taken

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from patients who were 11 ± 4 months old. None of the imperforate anus patients had HSCR. HSCR specimens were divided into aganglionic and ganglionic samples, and we compared the most distal aganglionic segments with the most proximal ganglionic segments. Tissue specimens were either snap-frozen in liquid nitrogen and stored at −80 °C for protein extraction or embedded in OCT Mounting Compound (VWR International, Leuven, Belgium) for immunofluorescence and stored at −80 °C until use.

1.2. Immunofluorescence staining and confocal microscopy

Frozen blocks of HSCR colon and normal control samples were sectioned transversely at a thickness of 10 μm, mounted on SuperFrost® Plus slides (VWR International, Leuven, Belgium) and fixed with 10% buffered formalin for 5 min. Sections underwent cell membrane permeabilization with 1% TritonX-100 for 20 min at room temperature. After blocking with 10% normal goat serum (Sigma Aldrich Ltd., Arklow, Ireland) for 30 min to avoid nonspecific absorption, sections were incubated with primary antibodies; mouse anti-NEDL2 and rabbit anti-ANO1 (Abcam), rabbit anti-PGP9.5 (Dako), all used at dilution 1:100, overnight at 4 °C. Sections were then washed in PBS + 0.05% Tween and incubated with corresponding secondary antibodies (goat ant-rabbit Alexa Fluor® 488, dilution 1:200 and goat antimouse Alexa Fluor® 647, dilution 1:200, Abcam, Cambridge, UK) for 1 h at room temperature. After washing, sections were counterstained with DAPI antibody, dilution 1:1000 (Roche Diagnostics GmbH, Mannheim, Germany) for 10 min, washed, mounted and coverslipped with Fluorescent Mounting Medium (DAKO Ltd., Cambridgeshire, UK). All sections were independently evaluated by two investigators with an LSM 700 confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany).

1.3. Protein extraction and Western blot

Specimens of HSCR colon and control colon were homogenized in RIPA buffer (Radio Immunoprecipitation Assay, Sigma-Aldrich Ltd., Wicklow, Ireland) containing 1% protease inhibitor cocktail (Sigma-Aldrich Ireland Ltd., Wicklow, Ireland). Protein concentrations were determined using a Bradford assay (Sigma-Aldrich Ireland Ltd., Wicklow, Ireland). A total volume of 20 μl Laemmli sample buffer (Sigma-Aldrich Ireland Ltd., Wicklow, Ireland) containing 10 μg of protein was loaded in the 10% SDS-PAGE gel (NuPAGE Novex Bis–Tris gels, Invitrogen, Carlsbad, USA) for electrophoretic separation. The electrophoresis was performed in MES SDS running buffer (Invitrogen, Carlsbad, USA). Proteins were then transferred to 0.45 μm nitrocellulose membrane (Millipore Corporation, Billerica, USA) by Western blotting. Following Western blotting, the membranes were blocked in 3% BSA–0.05% Tween for 30 min before antibody detection. Primary antibodies against mouse anti-NEDL2 and mouse anti-GAPDH (Abcam), dilution of 1:1000, were used and incubation was performed overnight at 4 °C. Following extensive washing (four times in PBS–0.05% Tween) the membranes were incubated with the appropriate secondary antibody (goat antimouse IgG-HRP, dilution 1:10,000, Abcam, Cambridge, UK), followed by washing (four times in PBS–0.05% Tween). Detection was performed with the ECL plus chemiluminescence kit (Thermo, Fisher Scientific, Dublin, Ireland). We used GAPDH (mouse anti-GAPDH, dilution 1:1000, Abcam, Cambridge, UK) as an additional loading control.

2. Results

2.1. Immunofluorescence staining and confocal microscopy

Immunohistochemistry in conjunction with confocal microscopy revealed NEDL2-positive cells within the mucosa, myenteric plexus and smooth muscle layers in normal controls and the ganglionic region of HSCR, with a marked reduction in NEDL2-positive cells in the same regions within the aganglionic HSCR specimens (Fig. 1). Immunofluorescent staining of NEDL2 distribution showed colocalization with ICCs and neurons.

2.2. Western blot

Western blotting revealed high levels of the NEDL2 protein in normal controls and the ganglionic region of HSCR, while there was a marked decrease in NEDL2 protein expression in the aganglionic region of HSCR (Fig. 2).

3. Discussion

Wei et al. have recently shown that mice lacking NEDL2 exhibit decreased numbers of enteric neurons, progressive bowel motility defects and intestinal hypoganglionosis [5]. The most striking feature of the NEDL2$^{-/-}$ mice is their perinatal lethality with perturbed intestine morphology; 100% of the NEDL2$^{-/-}$ mice die within 2 weeks after birth [5]. The authors observed impairment in the proliferation of NEDL2$^{-/-}$ ENS precursors; thus the number of ganglia was significantly reduced in these mice. This impairment was thought to be because of NEDL2's role as a positive regulator of GDNF/RET signaling [5]. The abnormalities reported in the NEDL2$^{-/-}$ mice are very similar to those observed in humans with HSCR.

The *RET* gene encodes a transmembrane receptor kinase, RET, that dimerizes when activated by a complex that includes a member of the glial cell derived neurotrophic factor (GDNF) family of ligands and a preferred glycosylphosphatidylinositol-anchored co-receptor, GDNF family receptor alpha (GFRα) [6]. GDNF/GFRα is crucial for ENS development. ENS precursor cells originate within the neural crest and migrate to the gut during the early stages of embryonic development. Through Ret, GDNF/GFRα1 stimulates the proliferation of neural crest cells, their migration, survival and differentiation [6]. ENS precursor proliferation is required to increase the ENS cell number to fully populate the progressively expanding gastrointestinal tract.

HSCR is a heterogeneous genetic disorder with dominant, recessive and polygenic forms, and a higher incidence is associated with a number of other genetic disorders including Down's syndrome [7]. It is characterized by the absence of ganglion cells in the distal bowel. The absence of ganglion cells in HSCR is attributed to a failure of migration of neural crest cells. The earlier the arrest of migration, the longer the aganglionic segment is. The aganglionosis is confined to the rectosigmoid area in more than 80% of patients. In the remaining patients, the aganglionosis extends beyond the rectosigmoid area involving the descending colon and transverse colon, or it may involve the entire colon along with a short segment of terminal ileum. Several genes are known to be involved in the development of HSCR. The RET gene, encoding a tyrosine-kinase receptor, is thought to be the major gene causing HSCR [8,9].

A recent meta-analysis by our group summarized the findings from 23 studies concerning RET with 1270 individuals affected with HSCR [10]. 228 (18%) of these HSCR cases were RET+. Of these 228, 96 (42%) presented as rectosigmoid, 81 (36%) long segment, 18 (8%) as TCA, 16 (7%) as total intestinal aganglionosis and 17 (7%) individuals were RET+ but no extent of aganglionosis was reported. In the rectosigmoid group, no significant association between phenotype and RET mutation could be shown, whereas a clear association could be shown between long segment disease, total colonic and total intestinal aganglionosis and RET mutations [10]. Mutations most often occurred in Exon 13 and showed significant association with rectosigmoid disease. No significance could be shown between RET+ and sporadic cases, albeit a trend towards RET+ and familial cases was observed [10]. One limitation of our current study is that we have not genotyped the HSCR patients, so we are unaware if any of these patients had RET mutations.

The results of our current study revealed colocalization of the NEDL2 protein with ICCs and neurons in the human colon. NEDL2 expression