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Altered expression of retinoblastoma 1 in Hirschsprung's disease

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ABSTRACT

Purpose: The retinoblastoma 1 (RB1) tumor suppressor is a critical regulator of cell cycle progression and development, and has been widely documented to be inactivated in human cancer. A recent study using RB1 knockout mice suggested a new role for RB1 in the normal regulation of the enteric nervous system (ENS), because of knockout mice showing ENS abnormalities and severe intestinal dysmotility. The aim of our study was to investigate the expression of RB1 in the normal human colon and in Hirschsprung's disease (HD).
Materials and methods: HD tissue specimens (n = 10) were collected at the time of pull-through surgery, while colonic control samples were obtained at the time of colostomy closure in patients with imperforate anus (n = 10). Immunolabeling of RB1 was visualized using confocal microscopy to assess protein distribution, while western blot analysis was undertaken to quantify RB1 protein expression.
Results: Immunohistochemistry revealed RB1 co-localized with platelet derived growth factor receptor alpha-positive (PDGFRα$^+$) cells, nitrergic neurons and glia in controls and the ganglionic region of HD, with a marked reduction in the aganglionic HD specimens. Western blotting revealed a marked decrease in RB1 protein expression in the aganglionic region of HD colon compared to ganglionic and normal controls.
Conclusion: We provide evidence of the presence of RB1 expression in the human colon in HD. As RB1 is known to colocalize with nitrergic neurons, the decreased expression of RB1 in the aganglionic bowel is most likely a secondary phenomenon because of the deficient nitrergic innervation in HD.

The enteric nervous system (ENS) is a network of neurons and glia that lies within the walls of the gastrointestinal tract. This is the largest and most complex division of the peripheral nervous system, containing more neurons than the spinal cord. Interstitial cells of Cajal (ICCs) are the pacemaker cells of the gut, producing spontaneously active pacemaker currents which drive the spontaneous electrical and mechanical activities of smooth muscle cells. Platelet-derived growth factor receptor alpha-positive (PDGFRα +) cells are another type of interstitial cell, which are also involved in neurotransmission, particularly purinergic, and lie in close proximity to enteric neurons and ICCs. Together with smooth muscle cells, these cells communicate with each other to regulate peristalsis and secretory activities of the gut. Hirschsprung's disease (HD) is a congenital condition characterized by the absence of nerve cells in the distal colon. HD is thought to be caused by the early arrest of neural crest cell migration during the embryonic development.

The retinoblastoma 1 (RB1) gene was first identified as the tumor suppressor gene mutated in retinoblastoma, a rare childhood cancer. The RB1 protein belongs to a family of three proteins, with other members being retinoblastoma-like protein 1 (RBL1) and retinoblastoma-like 2 (RBL2). These proteins are both functionally and structurally similar and are involved in the same pathways, but RBL1 and RBL2 show different functions with respect to RB1 in specific contexts [1–3]. RB1 knockout mice have recently been shown to die prematurely with symptoms of intestinal pseudoobstruction. These mice have serious defects in ENS structure and intestinal contractility but have a normal complement of myenteric neurons [4]. Thus, the aim of our study was to investigate the expression of RB1 in the normal human colon and in Hirschsprung's disease (HD).

1. Materials and methods

1.1. Tissue samples

This study was approved by the Ethics Medical Research Committee, Our Lady's Children's Hospital, Dublin, Ireland (Ref. GEN/292/12) and tissue samples were obtained with informed parental consent. HD specimens from 10 patients who underwent pull-through surgery were studied. These specimens were divided into aganglionic and ganglionic specimens. We compared the most distal aganglionic segments with the most proximal ganglionic segments. HD 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 from patients who were 11 ± 4 months old. None of the imperforate anus patients had HD. These specimens were divided into aganglionic and ganglionic samples.

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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 HD 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 non-specific absorption, sections were incubated with primary antibodies; rabbit anti-RB1, mouse anti-Ano1, mouse anti-nNOS and mouse anti-S100 (Abcam, Cambridge, UK), mouse anti-HuC/HuD (Molecular Probes), 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 anti-rabbit Alexa Fluor® 488, dilution 1:200 and goat anti-mouse 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 a LSM 700 confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany).

1.3. Protein extraction and western blot

Specimens of HD 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 rabbit anti-RB1, mouse anti-Ano1, mouse anti-nNOS and mouse anti-S100 (Abcam, Cambridge, UK), mouse anti-HuC/HuD (Molecular Probes), dilution of 1:1000, were used and incubation was performed overnight at 4oC. Following extensive washing (four times in PBS-0.05% Tween) the membranes were incubated with the appropriate secondary antibodies (goat anti-rabbit IgG, HRP-linked Antibody, dilution 1:10,000, and goat anti-mouse IgG-HRP, dilution 1:10,000, Abcam, Cambridge, UK) respectively 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.

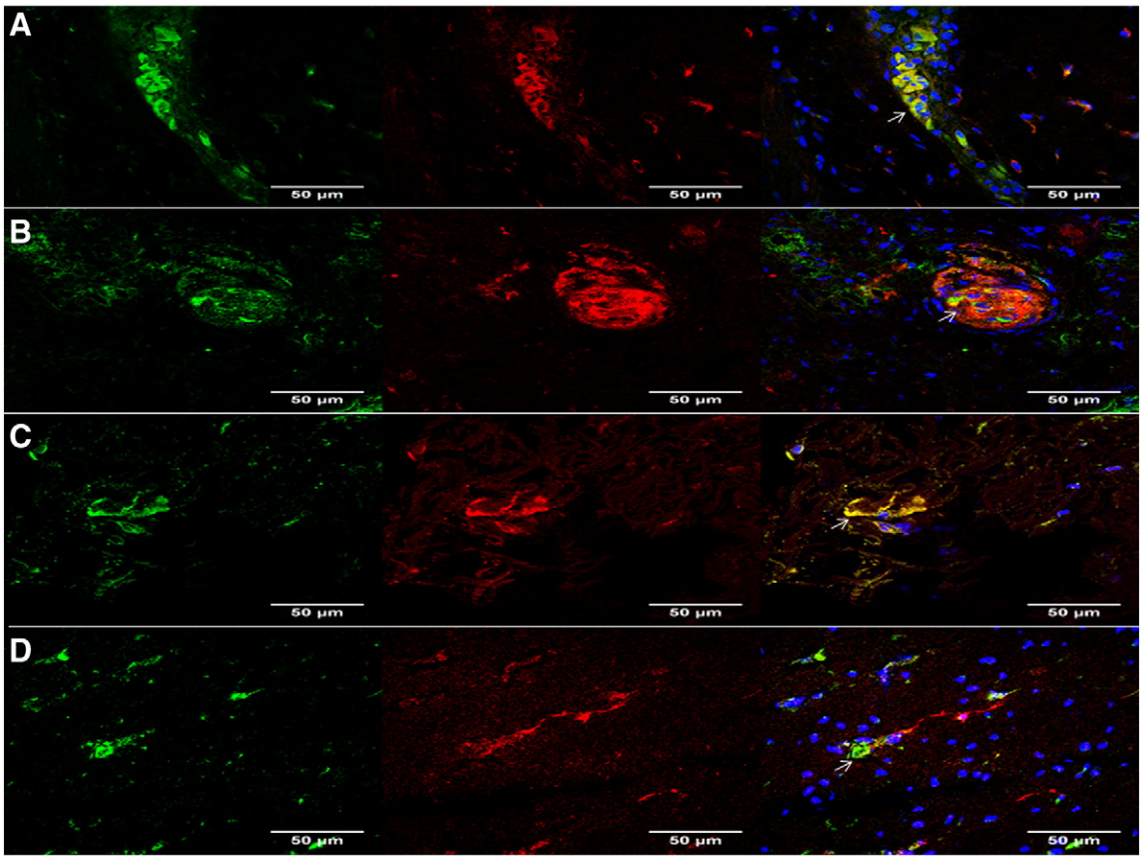

Fig. 1. Immunofluorescent staining of RB1-positive-cells (green), co-localized with (A) PDGFRα (red) labeling PDGFRα$^+$ cells, (B) nNOS (red) labeling nitrergic neurons and (C) S100 (red) labeling glia in ganglionic HD colon. No co-localisation was evident with (D) ANO1 (red) which labels interstitial cells of Cajal. Nuclei were stained with DAPI (blue). Arrows show co-localisation.