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ORIGINAL ARTICLE

Altered goblet cell function in Hirschsprung's disease

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Abstract

Aims and objectives Hirschsprung's disease-associated enterocolitis (HAEC) is the most serious complication of Hirschsprung's disease (HSCR). HAEC occurs in 17–50% of patients with HSCR and may occur before or after a properly performed pull-through operation. The pathogenesis of HAEC is poorly understood. It is well recognized that a complex mucosal barrier protects, as the first line of defense, the surface of healthy intestinal tract from adhesion and invasion by luminal micro-organisms. Within the intestinal epithelium, goblet cells secrete gel-forming mucins, the major component of mucus, which block the direct attachment of commensal bacteria to the epithelial layer. Mucin 2 (MUC2) is the predominant mucin expressed in humans. Trefoil factor 3 (TFF3) synergizes with mucin and enhances the protective barrier properties of the mucus layer. SAM pointed domain-containing ETS transcription factor (SPDEF) drives terminal differentiation and maturation of secretory progenitors into goblet cells. Krueppel-like factor 4 (KLF4) is a goblet cell-specific differentiation factor in the colon and controls goblet cell differentiation and activates mucin synthesis. We hypothesized that the goblet cell function in the ganglionic pulled-through bowel in HSCR is abnormal and, therefore, we investigated the changes in goblet cell differentiation and functional expression of mucin in the bowel specimens from patients with HSCR.

Material and methods We investigated MUC2, TFF3, SPDEF and KLF4 expression, and the goblet cell population in the ganglionic and aganglionic bowel of HSCR patients ($n = 10$) and controls ($n = 10$) by qPCR, Western blotting, confocal immunofluorescence, and alcian blue staining.

Results The qPCR and Western blotting analysis revealed that TFF3, SPDEF and KLF4 expressions were significantly downregulated in the aganglionic and ganglionic colon of patients with HSCR as compared to controls ($p < 0.05$). Alcian blue staining revealed that the goblet cell population was significantly decreased in aganglionic and ganglionic colon as compared to controls ($p < 0.05$). Confocal microscopy revealed a markedly decreased expression of TFF3, SPDEF and KLF4 in colonic epithelium of patients with HSCR as compared to controls.

Conclusion This is, to our knowledge, the first report of decreased expression of TFF3, SPDEF, KLF4, and goblet cell population in the colon of patients with HSCR. Altered goblet cell function may result in intestinal barrier dysfunction contributing to the development of HAEC.

Keywords Hirschsprung's disease · Hirschsprung's disease-associated enterocolitis · Goblet cell

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Introduction

Hirschsprung's disease (HSCR) is the most common congenital gut motility disorder and relatively common cause of intestinal obstruction in the newborn [1–3]. The gold standard treatment of HSCR is the pull-through operation,

which follows the principle of excision of the abnormally innervated, spastically contracted aganglionic colon and associated transition zone, with anastomosis of the normo-ganglionic bowel to a point just proximal to the dentate line in the anal canal. However, it is well recognized that despite having an optimal pull-through operation for HSCR, many patients continue to have persistent bowel symptoms. Hirschsprung's disease-associated enterocolitis (HAEC) is the most serious complication of HSCR and is the leading cause of disease-related morbidity and mortality. HAEC occurs in 17–50% of patients with HSCR and may occur before or after a properly performed pull-through operation [4]. The pathogenesis of HAEC is poorly understood. Several theories have been proposed including intestinal epithelial barrier dysfunction, abnormal innate immunity and disturbed microbiota composition.

It is well recognized that a complex mucosal barrier protects, as the first line of defense, the surface of healthy intestinal tract from adhesion and invasion by luminal micro-organisms. Goblet cells are specialized secretory cells found throughout the mucosal epithelium and considered as the gatekeepers of the mucosal immune system. Goblet cells play a key role in intestinal epithelial function by secreting gel-forming mucins, the major component of mucus, which blocks the direct attachment of commensal bacteria to the epithelial layer. The importance of goblet cells in the protection of intestinal mucosa is evident in the roles of goblet cell-derived mucin 2 (MUC2), a major gel-forming mucin and the small peptide, trefoil factor 3 (TFF3) [5]. TFF3 synergizes with mucin and enhances the protective barrier properties of the mucus layer.

SAM pointed domain-containing ETS transcription factor (SPDEF), is a member of the ETS transcription factor family, which is expressed in several organs including small and large intestinal epithelium [6]. In the intestine, SPDEF drives terminal differentiation and maturation of secretory progenitors into goblet cells. Krueppel-like factor 4 (KLF4) is a zinc-finger transcription factor expressed in the epithelium of several organs including intestine [7]. KLF4 is a goblet cell-specific differentiation factor in the colon and controls goblet cell differentiation and activates mucin synthesis.

As mentioned above, many patients continue to have recurrent episodes of HAEC despite having undergone a technically optimal pull-through operation. We hypothesized that goblet cell function in the ganglionic pulled-through bowel in HSCR is abnormal and, therefore, we investigated goblet cell differentiation and functional expression of mucin in the normoganglionic and aganglionic bowel specimens from patients with HSCR.

Materials and methods

Tissue collection

Ethical approval for collection of specimens was in place from both the institutions participating in the study (Our Lady's Children's Hospital Ethics Committee, GEN292.12; Temple Street Children's University Hospital Research and Ethics Committee, 13.003). Full-length resected bowel specimens obtained during pull-through operation for HSCR were collected. Resected tissue included aganglionic and ganglionic segments. Healthy control colonic specimens were obtained from the proximal colostomy limb at the time of stoma closure in patients with imperforate anus (anorectal anomalies). Tissue specimens were stored in three ways following collection. One segment of each specimen was fixed in formalin at room temperature, for paraffin embedding and immunochemistry. A second segment was snap-frozen in a mould containing optimal cutting temperature (OCT) medium and stored at − 80 °C for further using immunofluorescence and confocal microscopy. The remaining segment was stored at − 80 °C for RNA extraction and protein extraction.

RNA isolation

TRIzol reagent (Invitrogen) was used for the acid guanidinium-thiocyanate-phenol-chloroform extraction method to isolate total RNA from HSCR and control tissues ($n = 10$ for each group) according to the manufacturer's protocol. Spectrophotometrical quantification of total RNA was performed using a NanoDrop ND-1000 UV–Vis spectrophotometer (Thermo Scientific Fisher, Wilmington, USA). The RNA solution was stored at − 20 °C until further use.

cDNA synthesis and quantitative polymerase chain reaction

Reverse transcription of total RNA was carried out at 85 °C for 3 min (denaturation), at 44 °C for 60 min (annealing) and at 92 °C for 10 min (reverse transcriptase inactivation) using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, West Sussex, UK) according to the manufacturer's instruction. The resulting cDNA was used for quantitative real-time polymerase chain reaction (qRT-PCR) using a LightCycler 480 SYBR Green I Master (Roche Diagnostics, Mannheim, Germany) in a total reaction mix of 20 µl per well. The following gene-specific primer pairs were used: Human SPDEF (Eurofins) sense primer 5′ CGC TCC ATC CGC CAG TAT TA and Human SPDEF (Eurofins) antisense primer 5′ GTG CAC GAA CTG GTA GAC

GA, as well as Human MUC2 (Eurofins) sense primer 5′ ATC AAG CTG GCT CCC TCC TA and Human MUC2 (Eurofins) antisense primer 5′ TTC CAG CTG TTC CCG AAG TC, as well as Human KLF4 (Eurofins) sense primer 5′ AAC GAT CTC CTG GAC CTG GA and Human KLF4 (Eurofins) antisense primer 5′ ATA GGT GAA GCT GCA GGT GG, as well as Human TFF3 (Eurofins) sense primer 5′ GAC CAT GAA GCG AGT CCT GA and Human TFF3 (Eurofins) antisense primer 5′ ATC CTG GAG TCA AAG CAG CA. For normalization purposes, real-time RT-PCR was performed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH sense primer 5′ACA TCG CTG AGA CAC CAT GG and GAPDH antisense primer 5′ GAC GGT GCC ATG GAA TTT GC were used. After 5 min of initial denaturation at 95 °C, 55 cycles of amplification for each primer were carried out. Each cycle included denaturation at 95 °C for 10 s, annealing at 60 °C for 15 s, and elongation at 72 °C for 10 s. Relative mRNA levels of gene expression were determined using a LightCycler 480 System (Roche Diagnostics) and the relative changes in gene expression level of interest were normalized against the level of GAPDH gene expression in each sample (DDCT method). Experiments were carried out in duplicate for each sample and primer.

Protein extraction and Western blot

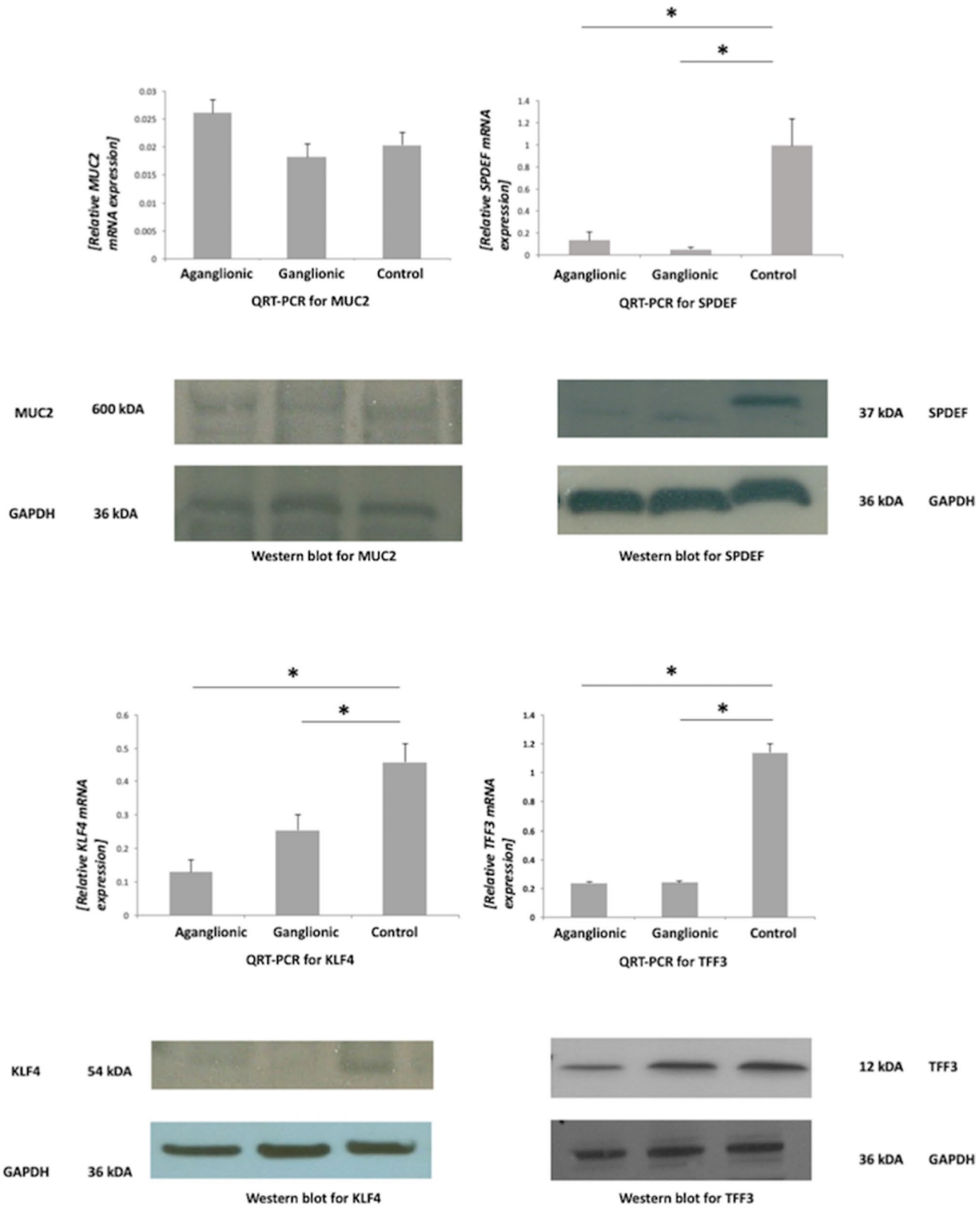
Specimens of HSCR colon and control colon were homogenized in RIPA buffer (Radio-Immunoprecipitation Assay, Sigma-Aldrich Ltd., Wicklow, Ireland). Protein concentrations were determined using a Bradford assay (Sigma-Aldrich Ireland Ltd., Wicklow, Ireland). A total volume of 40 µl Laemmli sample buffer (Sigma-Aldrich, Ireland Ltd., Wicklow, Ireland) containing 10 µg protein was loaded in the 10% SDS-PAGE gel (NuPAGE Novex Bis-Tris gels, Invitrogen, Garlsbad, USA) for electrophoretic separation. The electrophoresis was performed in MES SDS (2-(N-morpholino) ethane sulfonic acid, sodium dodecyl sulfate) running buffer (Invitrogen, Carlsbad, USA). Proteins were then transferred to a 0.45 µm nitrocellulose membrane (Millipore Corporation, Billerica, USA) by Western blotting. Following Western blotting, the membranes were blocked with 3% bovine serum albumin [BSA, Sigma-Aldrich, Ireland (A2153-50G)], for 60 min before antibody detection. The primary antibodies: rabbit anti-SPDEF (Abcam, Cambridge, UK, ab53881), rabbit anti-KLF4 (Abcam, Cambridge, UK, ab215036), rabbit anti-TFF3 (Abcam, Cambridge, UK, ab101099) and mouse anti-MUC2 (Abcam, Cambridge, UK, ab11197), dilution 1:1000, respectively, were used, and incubation was performed overnight at 4 °C. Following extensive washing (four times in PBS (phosphate-buffered saline)-0.05% Tween) the membranes were incubated with goat anti-rabbit IgG HRP-linked secondary antibody (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. Quantification of Western blot was conducted with ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland, USA) by measuring the density of each single band and normalizing it against the density of the corresponding GAPDH band.

Immunofluorescence

Colonic sections were embedded in OCT compound [VWR, Ireland (361603E)] and snap-frozen in liquid nitrogen. Twenty micron sections were cut and fixed in 10% neutral buffered formalin (Sigma-Aldrich, Ireland [HT501128-4L]). Cell membranes were permeabilized by rinsing in 1% w/v PBS with 1% Triton X-100. Sections were blocked in 10% BSA diluted in 1% w/v PBS with 0.05% PBST for 90 min at room temperature to prevent non-specific antibody binding. Samples were incubated simultaneously in both primary antibodies: rabbit anti-SPDEF (1:500, 5% BSA) (Abcam, Cambridge, UK, ab53881), rabbit anti-KLF4 (1:500, 5% BSA) (Abcam, Cambridge, UK, ab215036), rabbit anti-TFF3 (1:500, 5% BSA) (Abcam, Cambridge, UK, ab101099) and mouse anti-EpCAM (1:500, 5% BSA) (Santa Cruz, Heidelberg, Germany, sc-25308) at 4 °C overnight. Following incubation in primary antibody solution, samples were rinsed intensively in 0.05% PBST, following which they were incubated in a solution containing corresponding secondary antibodies (antirabbit Alexa Fluor488 (ab150073, GR226381), dilution 1:1000 and antimouse Alexa Fluor 584 (ab150116, GR232081), dilution 1:1000), (Abcam, Cambridge, UK) for 90 min at room temperature. After intensive rinsing in 0.05% PBST, samples were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) nuclear counterstain [Thermo Scientific, Ireland (EN62248)]. Sections were mounted with glass coverslips using Mowiol® 488 fluorescence mounting medium [Sigma-Aldrich, Ireland (81381-50G)], which was constituted according to the manufacturer's specifications. Specimens were visualized using laser scanning confocal microscopy (LSM700 confocal microscope, Carl Zeiss MicroImaging GmbH, Jena, Germany). Resulting images were processed using ImageJ, an open-access software available from http://imagej.nih.gov/ij/.

Alcian blue staining

Sections of 5 µm thickness were prepared from formalin-fixed, paraffin-embedded tissues. For goblet cell labelling, the sections were incubated in mixture of 3% aqueous



acetic acid and alcian blue for 10 min. After staining, the sections were washed in running water for 2 min. Counterstaining was performed with hematoxylin for 30 s followed by washing with water. After washing, the sections were put in acid alcohol for 3 s to differentiate. In addition, the sections were washed in running water briefly

◂**Fig. 1** qRT-PCR revealed significantly decreased relative mRNA expression levels of SPDEF, KLF4 and TFF3 in the aganglionic and ganglionic HSCR specimens ($n=10$) as compared to normal control tissue ($n=10$). Results are presented as mean ± SEM (*$p<0.05$ by one-way ANOVA). Western blotting revealed significantly decreased protein expression levels SPDEF, KLF4 and TFF3 in the aganglionic and ganglionic HSCR specimens ($n=10$) as compared to normal control tissue ($n=10$) ($p<0.05$ by one-way ANOVA). Equal loading of electrophoresis gels was confirmed by GAPDH staining

and then dehydrated and mounted on DPX using medite coverslipper.

Goblet cells per crypt were counted and normalized to total crypt length. At least three crypts per image and three images per section were examined from patient samples.

Statistical analyses

A one-way ANOVA was conducted to determine a statistically significance difference between aganglionic, ganglionic and healthy controls. Data are presented as mean +/− standard error. Specimens were classified into three groups: Aganglionic ($n=10$), Ganglionic ($n=10$) and healthy controls ($n=10$).

Results

Relative mRNA expression levels of MUC2, TFF3, SPDEF and KLF4.

The relative mRNA expression levels of TFF3, SPDEF and KLF4 were significantly decreased in aganglionic and ganglionic (HSCR) specimens as compared to normal controls ($p<0.05$, Fig. 1).

The relative mRNA expression levels of MUC2 were similar between the three tissue types ($p>0.05$).

Western blot

Our Western blot results from three independent experiments showed that TFF3, SPDEF and KLF4 protein was expressed in the colon of patients with HSCR and the expression was significantly decreased in the aganglionic and ganglionic bowel as compared to healthy controls ($p<0.05$, Fig. 1). The expression of MUC2 protein was not altered ($p>0.05$). Densitometry confirmed significantly decreased TFF3, SPDEF and KLF4 protein expression in the aganglionic and ganglionic bowel in HSCR as compared to normal controls. Equal loading of electrophoresis gels was confirmed by GAPDH (glyceraldehyde 3-phosphate dehydrogenase) staining of the stripped membranes.

Immunofluorescence staining and confocal microscopy

TFF3, SPDEF and KLF4 could be detected in the colonic epithelium of the aganglionic and ganglionic HSCR specimens and control samples. Compared with the control group there were significantly decreased expressions of TFF3, SPDEF and KLF4 in the colonic epithelium of patients with HSCR (Fig. 2).

Alcian blue staining

The goblet cell population was significantly decreased in aganglionic and ganglionic colon as compared to controls ($p<0.05$) (Fig. 3).

Discussion

To our knowledge, this is the first report describing significantly decreased expression of TFF3, SPDEF, KLF4, and goblet cell population in the colon of patients with HSCR. In the present study, qPCR and Western blotting analysis revealed that SPDEF and KLF4 were significantly downregulated in the aganglionic and ganglionic colon of patients with HSCR as compared to controls. Confocal microscopy revealed a markedly decreased expression of SPDEF and KLF4 in colonic epithelium of patients with HSCR as compared to controls. Alcian blue staining revealed that the goblet cell population was significantly decreased in aganglionic and ganglionic colon as compared to controls. Strikingly, the altered expression of TFF3, SPDEF and KLF4 was not only confined to the aganglionic colon but also affected the ganglionic colon which may explain why many patients continue to have episodes of HAEC and dysmotility problems despite a properly performed pull-through operation.

Proper functioning of the intestinal barrier between host and microbe is critical for health. Circumstantial evidence suggests that intestinal barrier dysfunction is associated with the pathogenesis of many forms of enterocolitis [8–10]. Components of the intestinal barrier include the lumen, the microclimate or mucus-containing layer, the epithelium, and the lamina propria. Each region plays a role in maintaining the intestinal barrier, and abnormalities seen in HSCR may contribute to the barrier dysfunction and the development of HAEC. One of the intestinal barriers is a microclimate made up of unstirred water and mucus predominantly composed of the MUC family of glycosylated proteins and produced by goblet cells within the lining of the epithelium. Mucus serves as a scaffold for bactericidal or bacteriostatic proteins that together create a primary barrier against bacterial invasion [8]. Immunoglobulins that have been secreted via transcytosis through the epithelial layer and defensins secreted by Paneth cells make up some of the antimicrobial proteins in

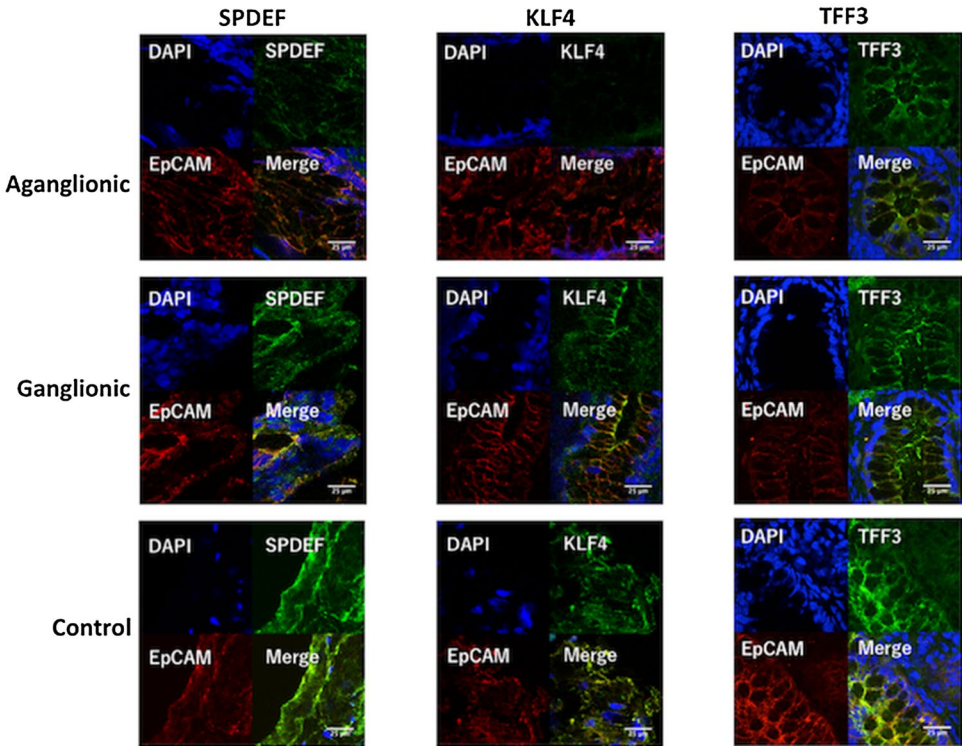


Fig. 2 SPDEF, KLF4 and TFF3 expression (green) in the colonic epithelium (red) of HSCR specimens as compared to normal controls. EpCAM (red) was used to identify colonic epithelium to show coexpression with SPDEF, KLF4 and TFF3 (scale bar 25 µm, original magnification ×63). SPDEF, KLF4 and TFF3 expression (green) in the colonic epitheium (red) of HSCR as compared to healthy controls. A total of eight specimens were analyzed; only the most representative stainings are shown. Compared with the control group there were significantly decreased expressions of SPDEF, KLF4 and TFF3 in colonic epithelium of patients with HSCR

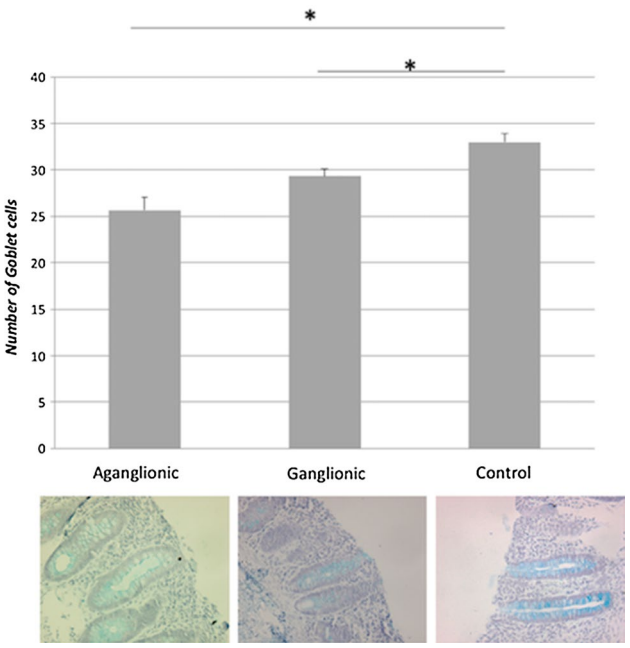


Fig. 3 Alcian blue staining revealed significantly decreased number of goblet cells in the aganglionic and ganglionic HSCR specimens as compared to normal control tissue. Results are presented as mean ± SEM (*$p < 0.05$ by one-way ANOVA)

this layer [11]. This layer has viscoelastic properties and acts as an adhesion site for bacterial and viral pathogens, preventing them from binding to epithelial cells [10, 12, 13]. An altered microclimate may lead to an increased susceptibility to infection. The maintenance of this defense is particularly important for the prevention of enterocyte adherence by the pathologic organisms. Enterocyte adherence allows organisms to invade epithelial barriers and makes the host susceptible to infection [14]. Deficient mucin secretion may predispose the host to the adherence of enteropathogenic organisms and contribute to the development of HAEC [15–17]. Abnormalities of the mucus protective barrier have been shown in the proximal ganglionated bowel after definitive surgery, which may contribute to the occurrence of post-pull-through enterocolitis and recurrent episodes of HAEC [18]. An abnormal mucin composition was identified in the pathologic specimens of HSCR [18].

Goblet cells are responsible for mucus secretion in the lumen of the gastrointestinal tract, which facilitates trapping of harmful bacterial and viral pathogens [19]. The large, highly glycosylated gel-forming mucin, MUC2, is the major component of the mucus in the intestine [20]. MUC2 polymers are densely packed in the regulated secretory vesicles of the goblet cell due to the low pH and high calcium in these compartments [19]. Small intestinal mucus fills the luminal space between the villi and usually also covers the villi tips, whereas the large intestine has a two-layered system [20]. The inner mucus layer is continuously formed by secretion from surface goblet cells [21]. Upon secretion, the MUC2 mucin unfolds and forms enormous net-like structures that are arranged by interacting with the previously secreted inner mucus layer [22]. A

recent study investigated goblet cell structure and function in a murine model of HAEC and demonstrated that both distal colon tissue from a murine model of HAEC and biopsies from HSCR patients showed similar expression of MUC2, in contrast to a previous study [23], which revealed that the expression of MUC2 was significantly decreased in the stool sample of HSCR patients as compared with control [18]. Similarly, in the present study, the expression of MUC2 was not significantly different in the aganglionic and ganglionic colon of patients with HSCR as compared with controls. It may be that functionally secreted levels of MUC2 may be altered, which would require more detailed analysis of colonic secretion systems.

TFF3 is another goblet cell-derived molecule belonging to a family of small cysteinerich secretory peptides that are expressed in a region-specific manner throughout the gastrointestinal tract [24]. TFF3 plays a critical role in wound healing by promoting epithelial restitution following mucosal injury [25]. In addition, TFF3 is thought to synergize with colonic mucins to enhance the protective barrier properties of the mucus layer against bacterial toxins [5, 26]. In the present study, the expression of TFF3 was significantly decreased in aganglionic and ganglionic colon of the patient with HSCR as compared with normal control.

SPDEF is required for proper goblet cell differentiation, maturation, and function in mucosal epithelia, making this transcription factor a focal point for studies of normal and abnormal goblet cell differentiation [6]. Loss of SPDEF results in impairment of goblet cell maturation in the intestine [27]. Conversely, over expression of SPDEF results in an expansion of goblet cells at the expense of other cell types in the intestine [6, 18]. It is clear that SPDEF plays an integral role in the differentiation and maturation of goblet cells [28]. SPDEF functions downstream of many common genetic pathways, including the Notch pathway, Wnt/ b-catenin/Tcf4 signaling, and TGF-b signaling, and is tightly regulated to maintain mucosal homeostasis [27]. KLF4 is a goblet cell-specific differentiation factor in the colon and controls goblet cell differentiation and activates mucin synthesis [7, 11]. In the colon, KLF4 deletion alters the terminal differentiation program of goblet cells [7]. Numbers of mature goblet cells relative to total epithelial cells are reduced by 90% in *Klf4*$^{-/-}$ mice as compared with littermate controls [7].

In summary, we report significantly decreased expression of TFF3, SPDEF, KLF4, and goblet cell population in the aganglionic and ganglionic colon of patients with HSCR. Our results suggest that altered goblet cell function in HSCR, may lead to intestinal barrier dysfunction and contribute to the development of HAEC.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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