

Original Article



Establishment and Characterization of Patient-Derived Intestinal Organoids from Pediatric Crohn's Disease Patients

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ABSTRACT

Purpose: This study aimed to establish and characterize patient-derived intestinal organoids (PDOs) from children with Crohn's disease (CD).

Methods: To generate PDOs, endoscopic biopsy specimens were obtained from non-inflamed duodenal bulbs of normal controls and CD patients. To verify the presence of PDOs, histological staining and quantitative reverse transcription polymerase chain reaction (RT-qPCR) analyses were performed.



Results: PDOs were successfully established in normal controls (n=2) and CD patients (n=2). Hematoxylin and eosin staining of formalin-fixed, paraffin-embedded PDO sections revealed crypt and villus structures, whereas immunofluorescence staining with EpCAM and DAPI confirmed the epithelial-specific architecture of the PDOs. RT-qPCR results revealed a significant increase in *Lgr5*, *Si*, and *Chga* gene expression and a decrease in *Olfm4* and *Muc2* expression in CD patients compared to normal controls, suggesting altered stem cell activity and mucosal barrier function ($p < 0.05$).

Conclusion: We successfully established and characterized PDOs in children with CD, providing a valuable tool for understanding the pathophysiology of the disease and evaluating potential therapeutic approaches. The differential gene expression of PDOs in CD patients might be caused by the complex interplay between epithelial adaptation and inflammation in the intestinal epithelium.

Keywords: Crohn disease; Organoids; Child; Biopsy; Endoscopy

INTRODUCTION

Crohn's disease (CD) is a chronic, relapsing-remitting inflammatory disease that affects the entire gastrointestinal tract. Owing to an incomplete understanding of the pathophysiology of the disease, pediatric cases present particular challenges [1]. The main treatment for CD involves biological therapies, such as antitumor necrosis factor agents, which are aimed at suppressing inflammation [2]. However, managing these therapies can be difficult, and success rates vary depending on factors such as disease severity and patient response to previous treatments [3]. In pediatric patients, biological therapies have been shown to induce clinical remission in approximately 40–60% of cases, with approximately 30–50%

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Conflict of Interest

The authors have no financial conflicts of interest.

maintaining remission over time [4]. While further research on the pathophysiology of CD is critical to overcome these challenges, studying pediatric patients presents additional limitations [5]. Thus, there is an urgent need to develop new treatments targeting different pathways to improve patient outcomes [6].

Conventional research models, such as in vitro cell cultures and in vivo animal models, have significant limitations [7]. Animal models have long life cycles, differ from humans in key biological aspects, and are costly, with additional concerns around animal ethics [8]. Similarly, cellular models often lack sufficient representation of human biology [9]. Consequently, more effective, safe, and human-representative cellular models are needed to reduce reliance on animal experiments. Patient-derived intestinal organoids (PDOs) offer a promising alternative because they recapitulate the structures, specific functions, molecular characteristics, and expression profiles of the primary intestinal mucosa [10]. These three-dimensional structures are derived from multipotent epithelial stem cells located at the base of intestinal crypts and are grown in culture from endoscopically obtained mucosal biopsies [11]. PDOs retain important features of the intestinal epithelium, such as self-renewal, self-organization, barrier function, and the ability to differentiate into various epithelial cell types [12]. As a result, PDOs have revolutionized research in inflammatory bowel diseases by providing new, patient-specific models for studying disease mechanisms and developing treatments [13,14].

Pediatric CD often leads to growth retardation, delayed puberty, and follows a different disease course compared to adult-onset CD [15]. These unique clinical features highlight the need for establishing pediatric-specific PDOs. Despite the growing incidence of CD in children, research addressing the specific characteristics of PDOs from pediatric patients remains sparse. This study aimed to establish and characterize PDOs in children with CD, providing a valuable tool for understanding the pathophysiology of the pediatric CD and evaluating potential therapeutic approaches.

MATERIALS AND METHODS

Collection of tissues and ethical statements

To generate PDOs, endoscopic biopsy specimens were obtained from the non-inflamed duodenal bulbs of both normal controls and CD patients at Seoul National University Hospital and Hallym University Kangnam Sacred Heart Hospital between November 2022 and May 2024. Normal controls were patients who exhibited normal mucosal findings during endoscopy. CD patients were diagnosed according to the pediatric CD guidelines [16]. All samples were collected after obtaining informed consent. This study was conducted in compliance with the Declaration of Helsinki and was approved by the Institutional Review Board of Seoul National University (approval no. H-2207-171-1344) and Hallym University Kangnam Sacred Heart Hospital (approval no. 2024-01-013). Written informed consent was obtained from all participants, and for participants under the age of 16 years, consent was obtained from their parents or legal guardians prior to the study.

Crypt isolation and passage

Intestinal crypts were isolated from fresh duodenal endoscopic biopsies using a modified protocol [17]. Briefly, biopsies (~4×7 mm) were thoroughly washed in PBS containing penicillin/streptomycin (Pen/Strep; Gibco) and amphotericin B (Gibco). The washes were

repeated until the solution became clear. The cleaned biopsies were then incubated in PBS supplemented with dithiothreitol (DTT; Sigma-Aldrich), ethylenediaminetetraacetic acid (EDTA; Invitrogen), and Pen/Strep for 30 minutes on ice. After incubation, the samples were shaken vigorously, and the supernatant was collected and supplemented with 1 mL of fetal bovine serum (FBS; Sigma-Aldrich). This process was repeated at least five times. All collected fractions were centrifuged at 1,000 rpm for 10 minutes at room temperature to pellet the crypts for further use. The collected cell pellet was resuspended in Matrigel, and 11.5 μ L drops of the Matrigel-cell suspension were pipetted into a 6-well plate. The culture medium was composed of advanced DMEM/F12 (Gibco), supplemented with pen/strep (Gibco), GlutaMAX (Gibco), non-essential amino acids (Gibco), Sodium Pyruvate (Gibco), N2 and B27 supplements (Sigma-Aldrich), Epidermal Growth Factor (EGF; PeproTech), Gastrin, N-acetyl cysteine, and insulin (Sigma-Aldrich). The culture medium was refreshed every two days, and the organoids were subjected to mechanical passaging at 7-to-8-day intervals.

Histologic analyses

Mature intestinal organoids were harvested by carefully collecting Matrigel, and the organoid pellet was fixed with 4% paraformaldehyde (Biosesang) and embedded in paraffin. Sections were cut at a thickness of 4 μ m for hematoxylin and eosin (H&E) and immunofluorescence (IF) analysis. The sections were deparaffinized with xylene and rehydrated using a graded ethanol series. For H&E staining, deparaffinized sections were stained with a H&E staining kit (Vector Laboratories) following the manufacturer's guidelines. For IF staining, the deparaffinized slides were subjected to antigen retrieval by heating in citrate buffer (Sigma-Aldrich) for 20 minutes. Endogenous peroxidase was inactivated with 0.3% hydrogen peroxide, followed by permeabilization with 0.15% TritonX-100 (Sigma-Aldrich) in PBS. Non-specific binding was blocked using goat serum (Sigma-Aldrich) before incubating the slides with primary antibody EpCAM (Cell Signaling Technology) overnight at 4°C. Slides were then incubated with a goat anti-mouse IgG (H+L) cross-absorbed secondary antibody, Alexa Fluor 488 (Invitrogen), for 1 hour. The nuclei were counterstained with DAPI (Invitrogen).

Quantitative reverse transcription polymerase chain reaction analyses

Total RNA from the organoids of normal controls and CD patients was isolated using TRIzol reagent (Invitrogen) and Direct-zol™ RNA Miniprep (Zymo Research), according to the manufacturer's protocol. Complementary DNA was synthesized using Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). mRNA expression levels were measured with FastStart Essential DNA Green Master (Roche), which was analyzed by using the Lightcycler® 96 Instrument (Roche). The primer sequences are listed in **Table 1**. Fold changes in gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method, with normalization to the housekeeping gene (18S ribosomal RNA).

Table 1. Quantitative reverse transcription polymerase chain reaction primer sequences

Gene	Forward	Reverse
18S	AGAAACGGCTACCATCCA	CCCTCAAATGGATCCTCGTT
Lgr5	CTCCCAGGTCTGGTGTGTG	GAGGTCTAGGTAGGAGGTGAAG
Olfm4	ACTGTCCGAATTGACATCATGG	TTCTGAGCTTCCACAAAATC
Si	TCCAGCTACTACTCGTGTGAC	CCCTCTGTTGGGAATTGTTCTG
Muc2	CAACAACACCCTGCTCAACG	CTTCGGGTCGCTCTTGAAGT
Chga	TAAAGGGGATACCGAGGTGATG	TCGGAGTGTCTCAAACATTCC
Lyz	CTTGTCCTCTTCTGTTACGG	CCCCTGTAGCCATCCATTCC

Statistical analysis

Statistical analysis of differences between groups was performed using Student's *t*-test from triplicate samples using GraphPad Prism (version 11; GraphPad Software, Inc.). A *p*-value of less than 0.05 was considered indicative of statistical significance.

RESULTS

Establishment of PDOs in pediatric CD patients

Normal and CD PDOs were established using endoscopic biopsy specimens obtained from non-inflammatory mucosal lesions in the duodenal bulb. Intestinal crypts were isolated from the duodenal biopsies of normal controls (*n*=2) and CD patients (*n*=2). **Table 2** shows the subject characteristics. Established PDOs from duodenal biopsies of normal controls were stably cultured for 23 weeks, and PDOs from non-inflamed duodenal biopsies of CD patients were stably cultured until 20 weeks before freezing. The PDOs were split once weekly, and the conditioned media were replaced three times a week. On average, a split ratio of 1:3–1:5 was used for the normal control and CD groups. **Fig. 1** shows the process of PDOs growth from days one to six in the fourth week of passage in the control group. From days one to three, the intestinal organoids began to recover from dissociation, showing early expansion and initial lumen formation (**Fig. 1**). By day six, the organoids had grown significantly and matured, with

Table 2. Clinical characteristics of subjects

Subjects	Age (yr)/sex	The reason of endoscopy	Endoscopic findings at the time samples acquisition	Diagnosis
Normal 1	13/M	Vomiting	NOS	Cyclic vomiting syndrome
Normal 2	13/F	Poor oral intake	NOS	Anorexia nausea
CD 1	15/F	Abdominal pain, chronic diarrhea, recurrent oral ulcer, weight loss	Multiple mucosal ulcers in the second portion of duodenum, and multiple cobblestone ulceration in colon.	CD Paris classification (A1b, L3+L4a, B1p, G0)
CD 2	12/F	Abdominal pain, poor oral intake, weight loss	Aphthous ulcers (jejunum on capsule and terminal ileum).	CD Paris classification (A1b, L1+L4b, B1, G1)

M: male, F: female, NOS: not otherwise specified, CD: Crohn's disease.

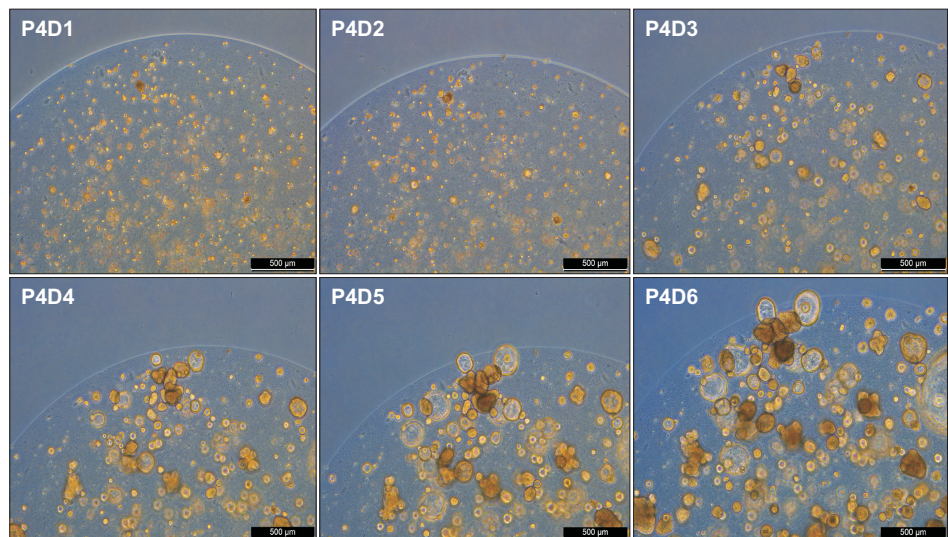


Fig. 1. Process of intestinal organoid growth from days one to six in fourth week passage in control group.

well-defined structures and differentiated cell types (**Fig. 1**). PDOs in CD patients exhibited the same growth pattern.

Histologic verification of PDOs

The histological structure of the PDOs on day seven using H&E staining is shown in **Fig. 2**, which revealed crypt and villus structures. Hematoxylin stains the nuclei of cells dark blue or purple, while eosin stains the cytoplasm and extracellular matrix pink. To confirm the growth of epithelial-specific organoids in our small intestinal crypt culture system, we immunostained the PDOs of normal control and CD patients for EpCAM and DAPI (**Fig. 3**).

Molecular characterization of PDOs in pediatric CD patients

In the RT-qPCR analyses, *Lgr5* gene expression was significantly higher in CD patients compared to normal controls ($p < 0.05$, **Fig. 4**), whereas *Olfm4* gene expression was significantly lower ($p < 0.05$, **Fig. 4**). Additionally, RT-qPCR showed a significant increase in *Si* and *Chga* gene expression and a decrease in *Muc2* expression in CD patients compared to normal controls ($p < 0.05$, **Fig. 4**). No significant differences in *Lyz* expression were observed (**Fig. 4**).

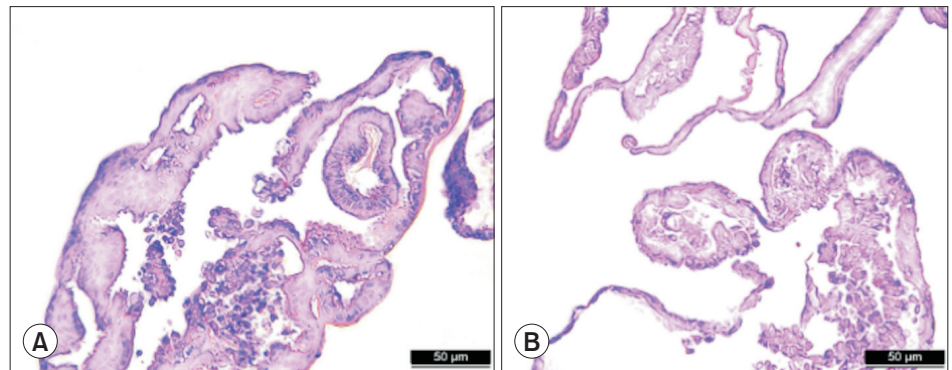


Fig. 2. Distinct crypt and villus structures in normal (A) and CD (B) samples visualized by hematoxylin and eosin staining from paraffin-embedded sections. Scale bars 50 μm . CD: Crohn's disease.

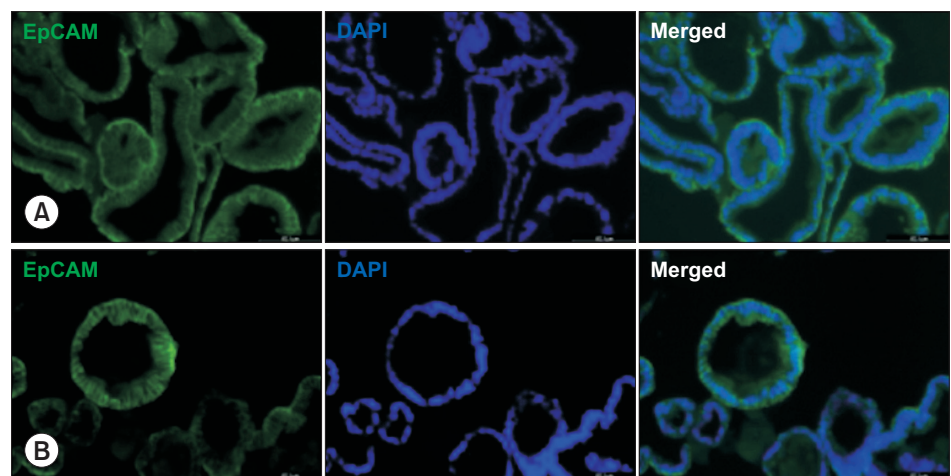


Fig. 3. Structural analysis of intestinal epithelial cells in human duodenal 3-dimensional organoids from control (A) and CD (B) patients using EpCAM immunofluorescence staining. Representative images show epithelial cell marker with EpCAM (green) and nuclei (blue) with DAPI. Scale bars 60 μm . CD: Crohn's disease.

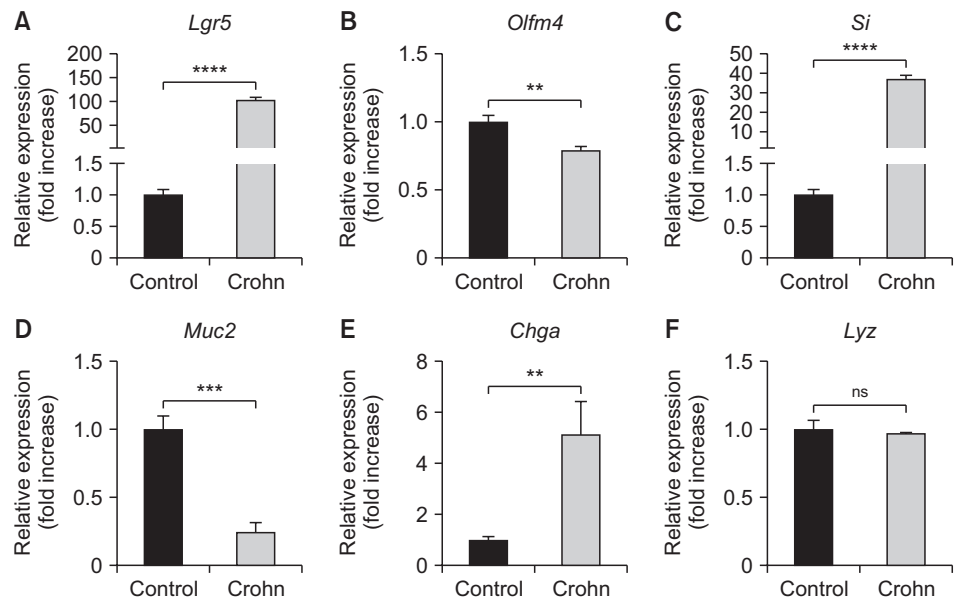


Fig. 4. mRNA expression of intestine-specific genes between normal and CD patients in human duodenal 3-dimensional organoids. mRNA expression level of stem cell-associated genes (A) *Lgr5* and (B) *Olfm4*, as well as intestinal epithelial maturation-associated genes (C) *Si*, (D) *Muc2*, (E) *Chga*, and (F) *Lyz* measured using quantitative reverse transcription polymerase chain reaction analyses. Gene expressions presented using $2^{-\Delta\Delta Ct}$ method. Statistical significance differences between groups analyzed by Student's *t*-test. ns: not significant, CD: Crohn's disease. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

DISCUSSION

This study describes the methodologies used to establish PDOs from the endoscopic biopsies of normal controls and CD patients. Our study characterized the specific gene expression profiles associated with CD, including increased expression of *Lgr5*, *Si*, and *Chga*, and decreased expression of *Olfm4* and *Muc2*, suggesting alterations in stem cell activity and mucosal barrier function. These findings provide valuable insights into the pathophysiology of CD and a new model for therapeutic research.

We determined that PDOs from both normal controls and CD patients accurately replicated the structural and cellular features of native intestinal tissue, as demonstrated by H&E and IF staining. EpCAM, a marker for epithelial cells, is commonly expressed on the basal or basolateral cell membrane in IF to indicate cell-to-cell adhesion in epithelial tissues, including intestinal organoids [18]. IF staining of EpCAM confirmed that the architecture of the established intestinal organoids closely resembled that of native intestinal tissues.

Additionally, RT-qPCR analyses revealed the expression of various gene markers in both PDOs, including *Lgr5* (stem cell marker), *Olfm4* (stem cell marker), *Si* (intestinal epithelial cell marker), *Muc2* (goblet cell marker), *Chga* (neuroendocrine cell marker) and *Lyz* (Paneth cell marker), indicating that they contain a diverse range of cell types [19].

In PDOs from CD patients, we observed notable differences in gene expression compared to PDOs from normal controls based on RT-qPCR results. Our study shows significantly increased *Lgr5* gene expression and decreased *Olfm4* gene expression in Crohn's PDOs compared with normal controls. Several mechanisms can explain these findings. Although *Lgr5* and *Olfm4* are stem cell markers, they are differentially regulated under different disease

conditions. *Lgr5* is crucial for maintaining intestinal stem cells and promoting epithelial regeneration, whereas *Olfm4* is associated with Paneth cells, which support stem cells but are less involved in their proliferation [20,21]. Chronic inflammation may cause changes in the intestinal epithelium and its microenvironment, thereby affecting stem cell markers. Additionally, increased *Lgr5* expression may reflect an adaptive response to damage, whereas decreased *Olfm4* expression may indicate impaired Paneth cell function [22]. Thus, the differential expression of *Lgr5* and *Olfm4* highlights the complex interactions between stem cell biology, inflammation, and tissue regeneration in CD.

A significant increase in *Si* and *Chga* gene expression and a significant decrease in *Muc2* gene expression were observed in PDOs from CD patients on RT-qPCR. Increased *Si* expression in CD likely represents an adaptive epithelial response, compensating for digestive function under stress [23]. This upregulation may also indicate changes in epithelial cell differentiation and tissue regeneration during inflammation [24]. Decreased *Muc2* expression in CD indicates a compromised mucosal barrier, because *Muc2* is crucial for protecting and lubricating the epithelial surface [25]. Lower *Muc2* levels suggest that inflammation disrupts the mucosal integrity, exacerbating epithelial damage and vulnerability [26]. Additionally, *Chga* gene expression analysis revealed increased chromogranin A, indicating a potential rise in neuroendocrine cell populations during inflammation. This upregulation may be an adaptive response of neuroendocrine cells to chronic stress or tissue damage [27]. Overall, these expression profile changes provide insights into the complex alterations in intestinal epithelial cell populations and their potential roles in CD pathology.

CD in children exhibits distinct clinical characteristics that differ significantly from those in adult populations [28]. Pediatric CD often leads to growth retardation, delayed puberty, and a different disease course compared to adult-onset CD [28]. Children with CD frequently present with more aggressive disease behavior, resulting in greater intestinal damage and higher rates of complications compared to adults [29]. Current literature emphasizes the unique pathophysiology and treatment responses in pediatric CD patients, highlighting the need for dedicated pediatric-oriented PDO settings [30]. However, research utilizing PDOs in children is lacking, particularly in Asian cohorts. Our established PDOs in pediatric CD can help advance research and improve clinical outcomes for children with CD.

Our study is the first report from Korea on the establishment of PDOs from mucosal biopsy samples of pediatric CD patients. By successfully generating organoids from patients, this study provides a groundbreaking model for investigating the pathophysiology of CD and predicting drug responses. The ability to perform such studies on organoids rather than directly on patients offers a novel approach for forecasting therapeutic responses. In this study, we performed RT-qPCR using various cell markers, and future experiments could include IF staining for these markers. Additionally, RNA sequencing could be employed to analyze genes and pathways specific to pediatric CD, paving the way for further insights. This study was limited by its small sample size. Our future plans include the creation of a CD organoid bank by collecting mucosal samples from a larger patient cohort.

In conclusion, we successfully established and characterized PDOs from pediatric CD patients, offering a valuable model for understanding the pathophysiology of the disease and evaluating potential therapeutic approaches. The differential gene expression of PDOs in CD patients might be caused by the complex interplay between epithelial adaptation and inflammation in the intestinal epithelium.

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