

Hath1 in a human intestinal epithelial cell line, LS174T cells. NICD is induced by DOX using a Tet-on system to mimic the acceleration of the Notch signal. NICD expression showed not only the decrease of Mucin2 (MUC2) expression but also a significant decrease of *Hath1* gene expression (Fig. 1A,B). We also assessed *Klf4* gene expression by NICD expression because *Klf4* is also essential to goblet cell differentiation.¹⁴ *Klf4* gene expression, however, was not affected by forced NICD expression (Fig. 1C), since it is suggested that the suppression of goblet cell phenotypic gene expression by Notch signaling is independent of *Klf4* expression.

To assess how Notch signaling suppresses the gene expression of *Hath1*, we selected the *Hes1* and *HeyL* genes as possible suppressors, based on previous identification of the *Hes* family genes induced by NICD in LS174T cells using a microarray system.⁸ We confirmed that the gene expression of *Hes1* and *HeyL* was markedly induced by NICD expression (Fig. 1D,E).

Hes1 But Not HeyL Suppresses *Hath1* Gene Expression in Human IECs, Resulting in the Decrease of MUC2 Gene Expression

To assess which genes suppress the *Hath1* gene expression, we generated cells (LS174T Tet-on *Hes1* cells and LS174T Tet-on *HeyL* cells) in which either *Hes1* or *HeyL* is induced by DOX using the Tet-on system, respectively. Forced expression of *Hes1* alone showed a significant decrease of MUC2 gene expression following the decrease of *Hath1* gene expression (Fig. 2A,B). In contrast, *HeyL* induction alone did not change the expression of either MUC2 (Fig. 2A) or *Hath1* genes (Fig. 2B). Moreover, neither *Hes1* nor *HeyL* induction affected *Klf4* gene expression (Fig. 2C). These results are compatible with previous reports that the depletion of *Hes1* in a mouse model upregulated *Atoh1* mRNA expression in intestinal epithelial cells, resulting in the hyperplasia of the goblet cells.¹⁵ Conversely, the finding that *Klf4* was not affected by the Notch signaling differs from previous reports.¹⁶

Hes1 Expression Alone Is a Sufficient Condition for the Repression of the Phenotypic Gene Expression of Goblet Cells by Notch Signaling

To further analyze the functional role of Notch signaling in the differentiation of IECs, we next asked whether *Hes1* expression alone is enough to compensate for the suppression of *Hath1* gene expression in Notch signaling. To inhibit the Notch signaling, LS174T Tet-on *Hes1* cells were treated with gamma-secretase inhibitor (GSI), which prevents the separation of NICD from the Notch receptor. Notch signal inhibition by GSI treatment alone showed a significant decrease of *Hes1* gene expression (Fig. 3A), in contrast to marked induction of MUC2

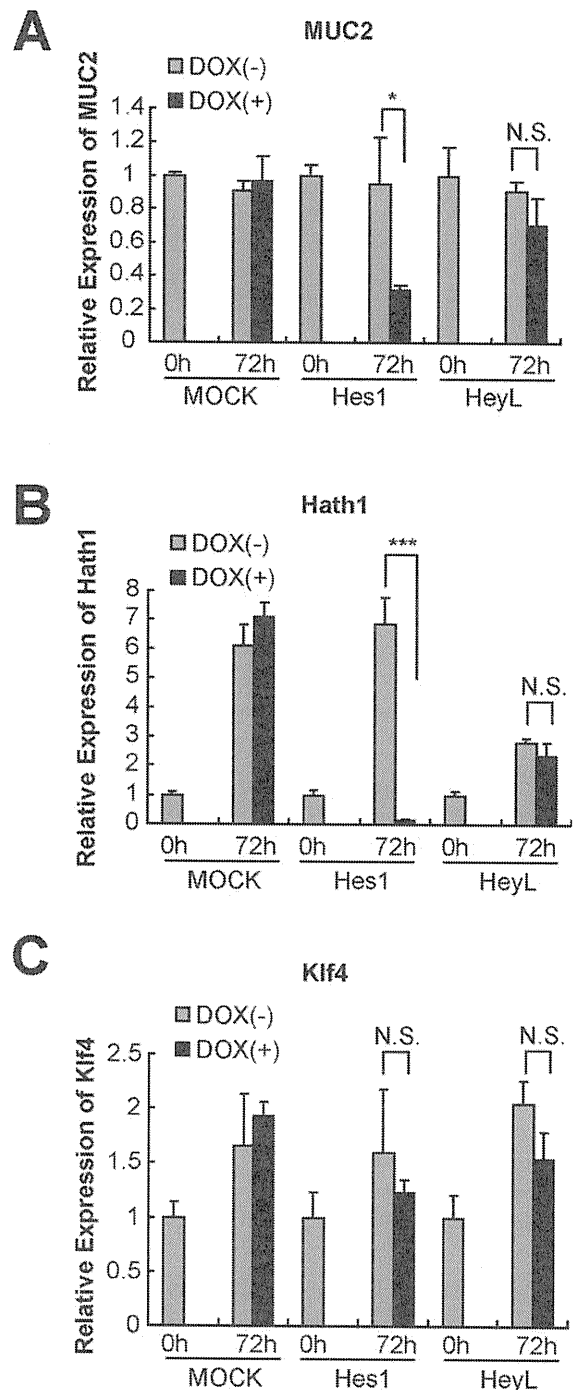


FIGURE 2. Gene alteration in LS174T cells by the expression of either *Hes1* or *HeyL*. (A) *Hes1* or *HeyL* was induced by DOX in LS174T Tet-on *Hes1* cells or LS174T Tet-on *HeyL* cells, respectively. *Hes1* induction significantly decreased MUC2 gene expression. (B) *Hes1* induction resulted in a significant decrease of *Hath1*. (C) Neither *Hes1* nor *HeyL* induction affected *Klf4* gene expression. (* $P < 0.05$, *** $P < 0.001$, $n = 3$).

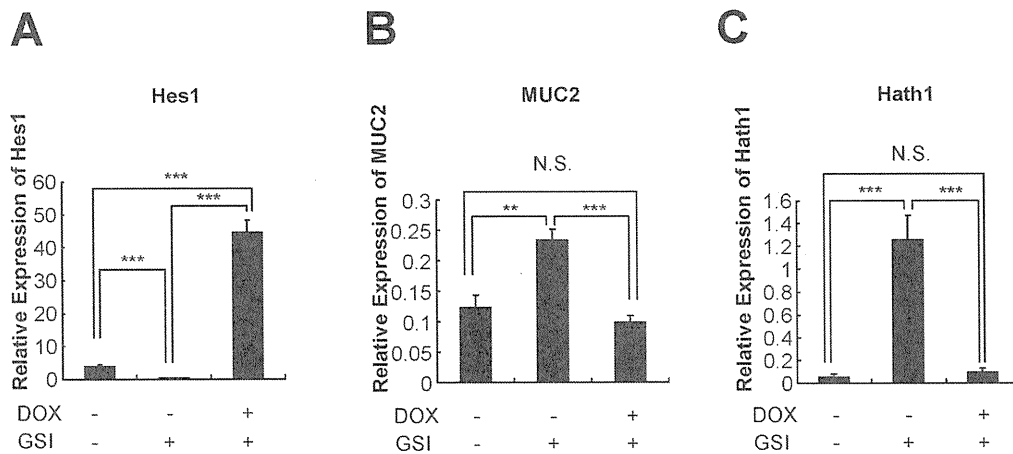


FIGURE 3. *Hes1* expression is enough to suppress intestinal cell differentiation by Notch signaling. (A) LS174T Tet-on *Hes1* cells were treated with GSI, which prevents the separation of NICD from the Notch receptor. GSI treatment alone significantly decreased *Hes1* gene expression. *Hes1* was induced by DOX in addition to GSI. (B) GSI markedly induced MUC2 gene expression. *Hes1* induction by DOX in GSI-treated cells restored MUC2 gene expression to the level in untreated cells. (C) GSI markedly induced *Hath1* gene expression. *Hes1* induction by DOX in GSI-treated cells restored *Hath1* gene expression to the level in untreated cells. (** $P < 0.01$, *** $P < 0.001$, $n = 3$).

gene expression (Fig. 3B) following the induction of the *Hath1* gene (Fig. 3C). Interestingly, the *Hes1* gene was expressed by DOX when Notch signaling was inhibited by GSI (Fig. 3A), while *Hath1* expression was restored to the level in untreated cells (Fig. 3C). Moreover, MUC2 gene expression was also decreased by *Hes1* expression alone (Fig. 3B).

These results indicate that *Hes1* might be a mainstream of Notch signaling to suppress the phenotypic gene expression of goblet cells in human intestine.

Previous results raised the question of whether *Hath1* is essential for expression of the MUC2 gene by Notch signaling inhibition. To assess the importance of the *Hath1* gene for MUC2 expression, the effect of silencing the *Hath1* gene using siRNA system was examined in LS174T cells in the Notch signaling-inhibited state. *Hath1* gene silencing resulted in cancellation of the *Hath1* gene expression induced by GSI treatment and restoration of MUC2 expression to the level in untreated cells (Supporting Information Fig. 1).

These results together suggest that Notch signaling affects the gene expression of *Hath1* but not *Klf4* to decide the fate of IECs.

HES1 Suppresses the Transcriptional Activity of *Hath1* Via the 5' Promoter Region

It has been reported that expression of *Math1*, the mouse homolog of *Atoh1*, was suppressed by *ZIC1* or *HIC1* via its 3' region.^{12,17} However, it has never been shown how *Hes1* suppresses the transcriptional activity of *Hath1* via Notch signaling. To assess the regulation of *Hath1* transcriptional activity, we constructed a reporter plasmid containing the 1000-bp upstream 5' region of *Hath1*. *Hath1* reporter plasmid was transfected into LS174T Tet-on *Hes1* cells or LS174T cells transfected with a mock plasmid. *Hes1* induction by DOX showed a significant decrease of the transcrip-

tional activity on *Hath1*, whereas the mock plasmid did not change its transcriptional activity (Fig. 4A). We then found three regions that matched the consensus sequence for binding *Hes1*, the Class C site,¹⁸ in the 1000-bp upstream region of *Hath1*. We therefore constructed a reporter plasmid in which all regions of the *Hes1* binding site in the 1000-bp upstream region of *Hath1* were deleted. As expected, reporter activity of the deletion mutant construct was not suppressed by *Hes1* expression. We next constructed mutants in which one of the binding sites of *Hes1* in the 1000-bp upstream region of *Hath1* was deleted. Interestingly, only the mutant construct lacking the second region of the *Hes1* binding site was not affected by *Hes1*, indicating that *Hes1* might directly suppress the *Hath1* transcriptional activity to bind to the second region of the *Hes1* binding site (Fig. 4A).

In chicken and mouse models, *Atoh1* expression is regulated only by the 3' region of *Atoh1* that contains both the enhancer region and the repressor region.^{12,19} We also found a homologous sequence of the enhancer region in the 3' region of *Hath1*, and a *Hes1* binding site in this enhancer region of *Hath1*. We therefore constructed a *Hath1* reporter plasmid containing the 3' region of *Hath1* behind the luciferase sequence. As before, *Hes1* suppressed *Hath1* transcriptional activity. Moreover, deletion mutants of the *Hes1* binding site in the 5' region of *Hath1* were also unaffected by *Hes1* expression, indicating that the *Hes1* binding site of the 3' region might not affect *Hath1* suppression by *Hes1* (Fig. 4B).

HES1 Binds Directly to the 5' Promoter Region of *Hath1*

To confirm the binding of *Hes1* to *Hath1* promoter region, we performed a ChIP assay. The region immunoprecipitated by *Hes1* antibody was amplified only in the 5' region

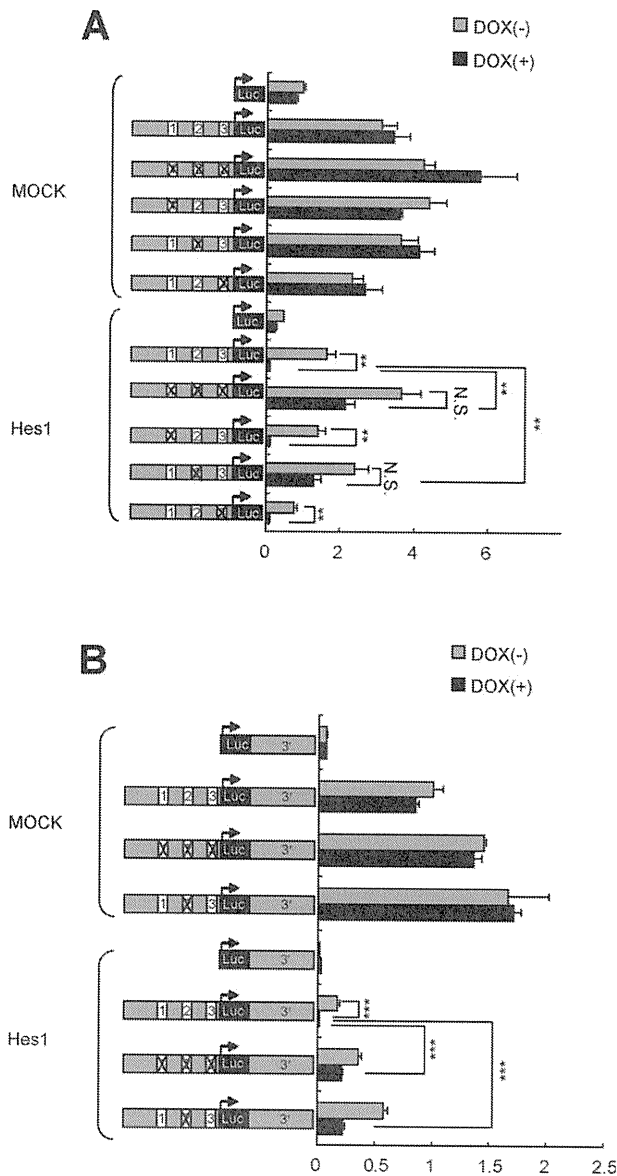


FIGURE 4. Hes1 regulates the transcriptional activity of Hath1 via 5' promoter region. (A) 5' Hath1 reporter plasmid containing the 1000-bp upstream region of Hath1 was transfected into LS174T Tet-on Hes1 cells and LS174T cells transfected with a mock plasmid. The induction of Hes1 by DOX significantly decreased the transcriptional activity on Hath1, whereas the transcriptional activity of the mock plasmid did not change. Three regions that matched the consensus sequence for binding Hes1, the Class C site, in the 1000-bp upstream region of Hath1 are indicated as square numbers. Reporter activity of a mutant with all regions of the Hes1 binding site deleted was not suppressed by Hes1 expression. A mutant construct in which only the second region of the Hes1 binding site was deleted was also unaffected by Hes1. (B) Hath1 reporter plasmid containing the 3' enhancer region of Hath1 behind the luciferase sequence was inserted into 5' Hath1 reporter plasmid. Hes1 also suppressed Hath1 transcriptional activity enhanced by 3' enhancer region. The deletion mutants of the Hes1 binding site in the 5' region of Hath1 were also unaffected by Hes1 expression (B). (** $P < 0.01$, *** $P < 0.001$, $n = 3$).

including the Hes1 binding sites but not 3' region of the Hes1 binding sites (Fig. 5B), supporting the idea that Hes1 binds directly to the 5' region of Hath1 to suppress the transcriptional activity in IEC.

Hes1 Does Not Completely Block the Transcriptional Activity of Hath1 Promoted by CDX2

To clarify the balance between the enhancer and the repressor in Hath1 transcriptional activity, we next assessed whether CDX2, which promotes *Atoh1* gene transcription in mice, is affected by Notch signaling on Hath1 transcription. Treatment with GSI showed slight induction of CDX2 in LS174T cells (Fig. 6A). Moreover, HES1 expression did not affect the expression of CDX2 (Fig. 6B), suggesting that the expression of CDX2 may be independent of Notch signaling. To assess the effect of CDX2 on Hath1 transcription regulated by HES1, a reporter assay of Hath1 was performed. Although CDX2 did not promote Hath1 transcription via the 5' promoter region of Hath1 (Fig. 6C), CDX2 cotransfected with the reporter plasmid containing the 3' enhancer region of Hath1 showed significant increase of transcriptional activity of Hath1 (Fig. 6D). Interestingly, the transcriptional activity of Hath1 promoted by CDX2 was not suppressed by Hes1 induction in LS174T tet-HES1 cells. These results suggest that Hes1 at the 5' region of Hath1 could not completely abrogate the transcriptional activity of Hath1 promoted via the 3' enhancer region by CDX2, and *Hath1* gene expression might be regulated by the balance between HES1 and CDX2.

Hath1 Protein Expression Is Decreased in the Goblet Cell Depletion of UC

We finally assessed whether Hath1 is decreased in colon mucosa with goblet cell depletion in line with the former results in vitro. In normal colonic mucosa, Hath1 and CDX2 were expressed in almost all IECs. In contrast, Hes1 was expressed in IECs situated in the lower half of the villi (Fig. 7). In UC patients, both Hath1 and CDX2 disappeared, while Hes1-positive cells were extended at the top of the villi (Fig. 7), indicating that the suppression of Hath1 in goblet cell depletion might be caused by both the disappearance of CDX2 and the extension of Hes1-positive cells.

DISCUSSION

This study reveals for the first time that Hes1 directly suppresses *Hath1* gene expression via the Notch signal, indicating that downregulation of Hath1 is associated with goblet cell depletion in human UC in combination with the disappearance of CDX2. Previous reports have suggested that Notch signaling suppressed the phenotypic gene expression of goblet cells by suppressing *Atoh1* gene expression,⁵ although it remains unknown how Notch signaling suppresses

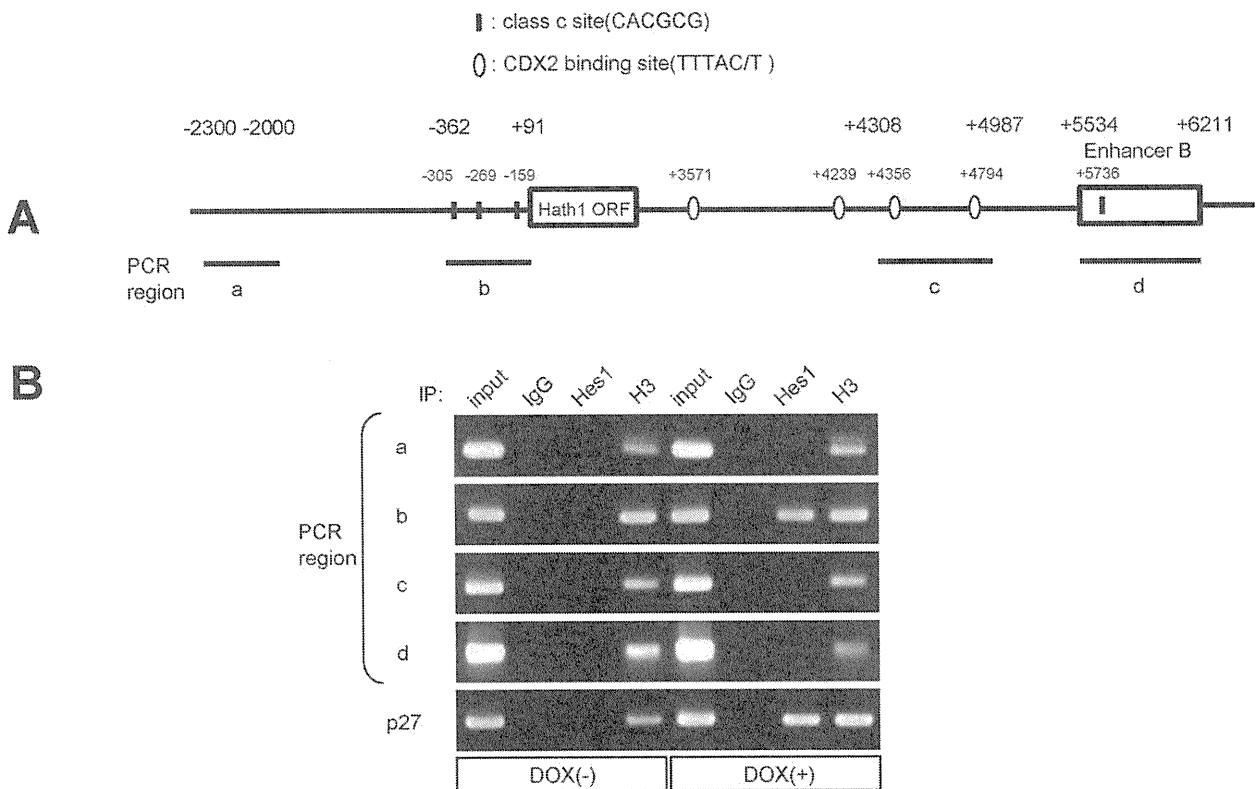


FIGURE 5. *Hes1* binds to 5' *Hath1* promoter region. (A) Schematic presentation of *Hath1* genome. (B) ChIP assay was performed using LS174T Tet-on *Hes1* cells with or without DOX treatment for 24 hours. Each region indicated by a letter in (A) was amplified from the immunoprecipitant by each antibody. The amplification of p27 from the immunoprecipitant by *Hes1* antibody was confirmed to be the known region of the *Hes1* binding site. Only the 5' region including the *Hes1* binding sites of *Hath1* (region b) was amplified from the immunoprecipitant by *Hes1* antibody under the induction of *Hes1* expression by DOX.

Hath1 gene expression. We first found that *Hes1*, but not *HeyL*, was necessary and sufficient for the suppression of *Hath1* gene expression by Notch signaling in IEC. Canonical Notch signaling leads to transcriptional activation of *Hes* family and *Hey* family genes such as *Hes1*, *Hes5*, *Hes7*, *Hey1*, *Hey2*, and *HeyL* by binding NICD to RBP-Jk.²⁰ *Hes* and *Hey* family genes play important roles in the differentiation of various tissues,^{21,22} but it has not been clarified how the function of each gene is assigned via Notch signaling. While we found that all *Hes* and *Hey* family genes were upregulated by NICD expression in intestinal cells, we also noticed that *Hes1* and *HeyL* were exorbitantly expressed by NICD than other *Hes* and *Hey* family genes (data not shown), suggesting that the functional assignment of Notch signaling is regulated by the quantity of each *Hes* and *Hey* family gene expressed. *HeyL* has been identified as one of the target genes of Notch3 receptor, because *HeyL* is expressed in smooth muscle cells of the digestive tract and the vasculature following Notch3 expression in later stages of development.²³ In this study, we could not identify the function of *HeyL* in goblet cell differentiation; rather, its function is expected to

be assessed in future study of the effect of Notch signaling on IEC.

On the other hand, we found that *Hes1* is critical for the differentiation into goblet cells via Notch signaling, since the binding of HES1 to the *Hath1* 5' promoter region silences *Hath1* gene expression. Although the 3' region of *Atoh1* has been characterized as the enhancer and repressor region to regulate *Hath1* gene expression by CDX2, *Zic1*, and *Hic1*, the function of the 5' region of *Atoh1* has not been clarified. This study revealed that the 5' region of *Hath1* is necessary not only for basic transcription but also for the regulation by HES1 via Notch signaling to presumably suppress the transcriptional activity of the basic transcription factors. It has been reported that *Hes1* binds not only to the N-box sequence but also to class C sites to suppress the expression of genes such as *P27^{kip1.18}* and *achaete-scute homolog-1*,²⁴ through which it plays a central role in cell proliferation and differentiation, respectively. In this study we identified a class C site at position -289 of the 5' region of *Hath1*, playing a crucial role in the regulation of *Hath1* gene expression by the Notch signal. We therefore suspected that *Hes1* might completely shut out the transcriptional activity via the

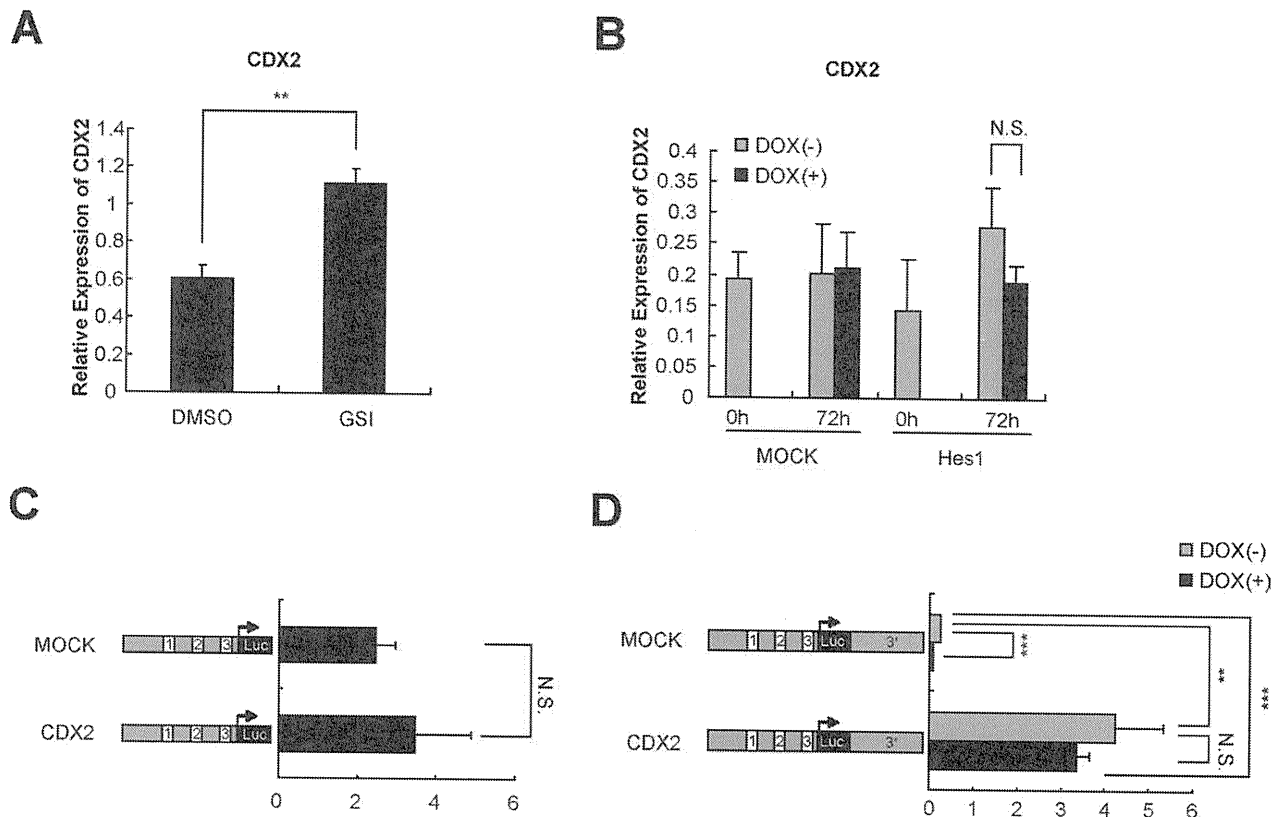


FIGURE 6. CDX2 enhances the transcriptional activity of Hath1 independently of Notch signaling. (A) *CDX2* gene expression was analyzed by treatment of LS174T cells with GSI for 72 hours. CDX2 was slightly upregulated by Notch signal inhibition. (B) *CDX2* gene expression was analyzed by the Hes1 expression induced by DOX in LS174T Tet-on Hes1 cells. CDX2 gene expression was not affected by Hes1 expression. (C) Transcriptional activity of Hath1 via the 5' region by CDX2 was assessed in LS174T cells for 72 hours after transfection of both the *CDX2* gene and 5' Hath1 reporter plasmid. CDX2 did not affect the transcriptional activity via the 5' promoter region of Hath1. (D) HES1 did not suppress the transcriptional activity via the 3' region of Hath1 by forced expression of CDX2. The transcriptional activity of Hath1 was assessed for 72 hours after transfection of both the *CDX2* gene and 3' Hath1 reporter plasmid with or without DOX in LS174T Tet-on HES1 cells. (** $P < 0.01$, *** $P < 0.001$, $n = 3$).

3' enhancer region, but that forced expression of CDX2 could induce the transcriptional activity of Hath1 even with Hes1 expression. Moreover, the expression of CDX2 was not affected by Notch signaling, suggesting that CDX2 and HES1 independently regulate *Hath1* gene expression. Thus, regulation by Hes1 via Notch signaling is not sufficient to suppress the gene transcription of *Hath1*, indicating that the transcriptional activity of Hath1 is regulated by the balance between CDX2 and HES1 expression.

Importantly, the present study also indicated that Hath1 is essential to regulate goblet cell formation in UC. Although the expression of Hath1 in inflamed mucosa of UC has been reported,²⁵ the correlation between goblet cell content and Hath1 expression in UC has not been elucidated. We confirmed that Hath1 was expressed in inflamed mucosa with conserved goblet cell formation in UC (data not shown), since goblet cell content might correlate with Hath1 expression in UC. In *Atoh1*-deficient mice, secretory lineages of IEC including goblet cells are completely lost,^{9,26} indicating that Hath1 might have the function of

not only mucus production but also differentiation toward goblet cells in human intestine.

Moreover, this study suggested that goblet cell depletion in UC caused by the disappearance of Hath1 required not only HES1 expression but also CDX2 suppression of IEC. CDX2 has been reported to be downregulated in UC mucosa,²⁷ but it remains unknown how CDX2 expression is suppressed by colonic inflammation even though CDX2 is upregulated by inflammation in the esophagus and stomach.^{28,29} One previous report indicated that CDX2 expression is suppressed by hypoxia inducible factor 1 (HIF1).³⁰ Another report found that HIF1 is overexpressed in UC mucosa,³¹ suggesting that HIF1 might suppress CDX2 expression in UC. Whatever the case, the regulation of CDX2 expression of IEC should be assessed to clarify the mechanism of goblet cell depletion in UC.

In conclusion, we have revealed for the first time that Hes1 is sufficient to suppress *Hath1* gene transcription via the Notch signal, but insufficient to suppress *Hath1* gene transcription by CDX2. The cooperation between Hes1 and

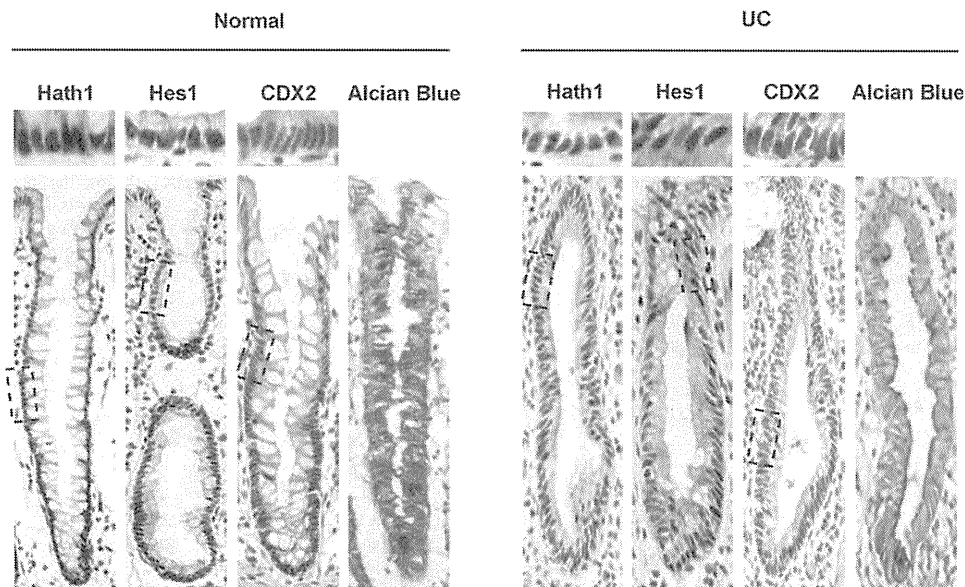


FIGURE 7. Immunohistochemistry of intestinal mucosa in UC. In normal colonic mucosa, *Hath1* and *CDX2* were expressed in most IEC. *Hes1* was expressed in intestinal epithelial cells in the lower half of villi. In UC mucosa with goblet cell depletion, neither *Hath1* nor *CDX2* was expressed, whereas *Hes1* was expressed up to the top of the villi. Upper column shows magnified view of the upper villus areas identified by dashed line in the lower column. Blue staining with Alcian blue represents goblet cells. The examination was performed by using the sections from three different individuals.

CDX2 is important to regulate *Hath1* gene expression, which is involved in goblet cell formation in UC. More detailed analysis of *Hath1* expression at various stages of UC or other enteritis diseases associated with goblet cell depletion will lead us understand the regulation of *Hath1* reduction under the inflammation state with various cytokines and inflammatory cells infiltration. Finally, elucidation of the mechanism of goblet cell depletion in UC will help us to develop novel therapies for strengthening the barrier function of colonic mucosa.

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Double-Blind, Placebo-Controlled Trial of Oral Tacrolimus (FK506) in the Management of Hospitalized Patients with Steroid-Refractory Ulcerative Colitis

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Background: We report a multicenter study of oral tacrolimus (FK506) therapy in steroid-refractory ulcerative colitis (UC).

Methods: In a placebo-controlled, double-blind study, 62 patients with steroid-refractory, moderate-to-severe UC were randomized into either a tacrolimus group or a placebo for 2 weeks. Patients were evaluated using the Disease Activity Index (DAI). As an entry criterion, patients had to have a total DAI score of 6 or more as well as a mucosal appearance subscore of 2 or 3. Clinical response was defined as improvement in all DAI subscores. Mucosal healing was defined as mucosal appearance subscore of 0 or 1. Clinical remission was defined as a total DAI score ≤ 2 with an individual subscore of 0 or 1.

Results: The mean total DAI score at study entry was 9.8 ± 1.61 in the tacrolimus group and 9.1 ± 1.05 in the placebo group. At week 2 the clinical response rate was 50.0% (16/32) in the tacrolimus group and 13.3% (4/30) in the placebo group ($P = 0.003$). The rate of mucosal healing observed was 43.8% (14/32) in the tacrolimus group and 13.3% (4/30) in the placebo group ($P = 0.012$) and the rate of clinical remission observed was 9.4% (3/32) in the tacrolimus group and 0.0% (0/30) in the placebo group ($P = 0.238$). The therapies in this study were well tolerated, with only minor side effects.

Conclusions: Oral tacrolimus therapy in patients with steroid-refractory UC shortened the acute phase and induced rapid mucosal healing. These results suggest that tacrolimus therapy is useful as an alternative therapy for steroid-refractory UC.

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Key Words: ulcerative colitis, immunosuppressive therapy, tacrolimus

Tacrolimus, a macrolide immunosuppressant produced by *Streptomyces tsukubaensis*, a species of *Actinomyces*, was discovered in 1984 on Mt. Tsukuba in Japan. Fellermann et al¹ reported the results of a study of tacrolimus in patients with steroid-refractory, severe ulcerative colitis (UC). With patients initially treated by continuous intravenous infusion and subsequently transferred to oral adminis-

tration, the study showed improved symptoms in five of six patients, with successful induction of remission and steroid tapering achieved in four patients. A report on oral and injectable formulations of tacrolimus stated, “most importantly, oral tacrolimus therapy appears to be effective and obviates the need for intravenous dosing.”²

Baumgart et al³ demonstrated the usefulness of low doses of oral tacrolimus (4–6 ng/mL) and Högenauer et al⁴ reported, “Oral tacrolimus might be an effective alternative treatment to intravenous cyclosporine for treatment of steroid-refractory UC.”

As no evaluation had yet been made of tacrolimus using a placebo as comparator, we conducted a dose-ranging study to evaluate oral administration over 2 weeks.⁵ The study established a placebo group, a group with a target tacrolimus trough concentration of 10–15 ng/mL, and a group with a target tacrolimus trough concentration of 5–10 ng/mL. The results indicated a significant difference in efficacy between the 10–15 ng/mL group and the placebo group over the short 2-week period.

Here we report on a multicenter study which was a double-blind study of oral administration for 2 weeks,

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comparing a placebo group with a group having a target tacrolimus trough concentration of 10–15 ng/mL.

MATERIALS AND METHODS

Patient Selection

Patients with moderate-to-severe, active UC were eligible for inclusion in this study. UC was defined according to standard criteria for symptoms and standard radiographic and endoscopic criteria.⁶ Before starting treatment, infectious diarrhea was ruled out by stool cultures and *Clostridium difficile* toxin testing. Endoscopies were performed during the week prior to the first dose of the study drug. The extent of colonic involvement was determined by total colonoscopy. All patients in the study had left-sided colitis and pancolitis and all were hospitalized.

Patients with known renal or severe hepatic dysfunction and pregnant women were excluded from the study. Pretreatment assessment included taking a history of the patient, physical examination, complete blood count, chemistry screening panel, and urinalysis.

Patients were classified as steroid-resistant or steroid-dependent. Patients with active UC were defined as steroid-resistant when the disease failed to respond to a systemic daily dose of 1 mg per kg of body weight, or 40 mg or more of prednisolone given over at least 7 days, or the equivalent of a daily dose of prednisolone of 30 mg or more over at least 2 weeks. Steroid-dependent patients were defined as patients with active UC in whom attempts to taper steroids had been unsuccessful. The steroid dosage remained the same from study initiation for 2 weeks, while only those patients in whom a dose of prednisolone of 60 mg/day or more was effective were permitted to decrease the dosage during this period. Efficacy was based on improvement in the frequency of stools and a decreased amount of blood in the stool.

Patients were evaluated using the Disease Activity Index (DAI).⁷ The DAI score is a sum of subscores for the following four factors: stool frequency, rectal bleeding, mucosal appearance, and physician's overall assessment, each of which is graded on a scale from 0 to 3. The DAI score ranges from 0 to 12; the higher the score, the more severe the disease activity. As an entry criterion, the patient was required to have a total DAI score of 6 or more, as well as a mucosal appearance subscore of 2 or 3.

Patients who started taking azathioprine within 3 months prior to entering the study were excluded from the study, and patients were permitted to continue taking azathioprine at an unchanged dose over the period beginning 3 months prior to the start of the study, until completion of the study. Patients were permitted to continue taking 5-aminosalicylic acid during the study, as long as the drug dosage was not changed over the period beginning 2 weeks prior to the start of the study, until completion of the study. Receiving cytapheresis within 14 days prior to entry in the study was a reason for exclusion

from the study. Patients receiving concomitant nutritional therapy continued to receive the same therapy during the study.

As UC therapy with cyclosporin, biological therapies, 6-mercaptopurine, or other immunosuppressants was not covered by health insurance in Japan, the concomitant use of these drugs was prohibited.

Protocol Review

The study protocol was reviewed and approved by each Institutional Review Board. Each patient read and signed a consent form before enrollment in the study.

Study Design

We conducted a multicenter study of oral tacrolimus treatment, consisting of a 2-week placebo-controlled, double-blind, randomized study in which patients with active UC were given either placebo or tacrolimus at an oral dose sufficient to achieve and maintain target blood concentrations of 10–15 ng/mL.

Open-label Extension

After week 2, patients received conventional treatment or tacrolimus open-label treatment. Data were collected during an open-label extension phase of the study. The effect of continuous treatment in the tacrolimus group was evaluated by comparing the condition of patients in the tacrolimus group at weeks 2 and 12.

Administration and Monitoring of Study Drug

The tacrolimus capsules used (Tacrolimus, Astellas Pharma, Japan) contained 0.5 mg or 1 mg of FK506. In consideration of safety, tacrolimus therapy was initiated at a small dose of 1–2.5 mg per time, twice daily. Dose adjustments were determined using proportional calculations of “blood trough concentration at steady state” and “target trough concentration” as shown in Table 1. To reach the target trough concentration quickly, the first dose adjustment occurred at an early stage. This increase required blood collection at 12 hours (C12h) and 24 hours (C24h) after the initial dose for determination of the trough concentration of tacrolimus in whole blood. Steady-state values were estimated to be 4 times the value at C12h, 2.5 times the value at C24h, or 3 times the mean value of C12h and C24h. The dose was adjusted by proportional calculation using a target concentration of 12.5 ng/mL. These equations were created based on the known pharmacokinetic profile of tacrolimus in healthy volunteers (data not shown).

For the next adjustment, measured values were checked against the target trough concentration. When the measured value was outside the range of 10–15 ng/mL, the dose was readjusted using blood trough concentration at steady state.

The randomization was performed by the Control Center (Bellssystem24, a third-party organization independent of study physicians and sponsor). To preserve blinding, blood trough

TABLE 1. Dose Adjustment of Tacrolimus

Dosage calculation method using trough concentration

Blood trough concentration under the same food intake condition as at administration should be used (fed/fasted condition).

For 2 weeks:

The dose is increased to a target trough concentration of 10-15 ng/mL (target of 12.5 ng/mL).

Initial adjustment (a, b, or c)

Initial dose

Weight (kg)	30 ≤ <50	50 ≤ <70	70 ≤ <90	90 ≤ ≤100
Dose per time (mg), twice daily	1	1.5	2	2.5

The blood trough concentration at 12 hours (C12h) and/or 24 hours (C24h) after the initial dose.

a: Initial dose (mg) × target trough concentration (12.5 ng/mL) / (average of C12h & C24h × 3).

b: Initial dose (mg) × target trough concentration (12.5 ng/mL) / (C12h × 4).

c: Initial dose (mg) × target trough concentration (12.5 ng/mL) / (C24h × 2.5).

Next adjustment:

The blood trough concentration (C) was measured at steady-state, after 2 days or more following the previous adjustment, to check whether the value was within the range of 10-15 ng/mL.

When the measured value was outside the range of 10-15 ng/mL, the dose was readjusted.

Previous dose × target trough concentration (12.5 ng/mL) / C.

levels were measured by SRL (a third-party organization independent of study physicians and sponsor) and relayed to the Control Center (Bellsystem24). Dosages were calculated at the Control Center based on the trough levels. The clinical sites were informed of the adjusted dosage by 3 days after the blood sample was drawn. Patient doses in the placebo group were pseudo-adjusted to preserve study blinding. The Control Center used the equations shown in Table 1 to carry out dose adjustments.

Symptom Assessment and Study Endpoints

The primary endpoint was clinical response based on the DAI score.⁷ Clinical response was defined as a reduction in DAI by at least 4 points and improvements in all categories (stool frequency, rectal bleeding, mucosal appearance, and physician's overall assessment). A worse or unchanged score in any category was considered a treatment failure, even if all other scores improved. Secondary endpoints were mucosal healing and clinical remission.⁸ Mucosal healing was defined as mucosal appearance subscore of 0 or 1. Clinical remission was defined as a total DAI score ≤2 with individual subscore (stool frequency, rectal bleeding, mucosal appearance, and physician's overall assessment) of 0 or 1. When a patient's symptoms worsened at any time and the investigator decided the study drug could not be continued, the treatment was considered a failure.

Statistical Analysis

Fisher's exact test was used to compare the tacrolimus group with the placebo group for demography, efficacy, and safety. The Wilcoxon signed rank test was used to compare each timepoint with baseline for demography. All statistical tests were two-sided with a significance level of 0.05 unless otherwise specified.

Sample Size

Based on previous results,⁵ the clinical response was assumed to be 50% in the tacrolimus group and 10% in the placebo group. We estimated that randomizing 31 patients to each group would be sufficient to show a difference in efficacy between placebo and tacrolimus based on the above assumptions and a two-sided alpha of 0.025 and power of 0.9 using a normal approximation.

RESULTS

Patient Population

This study was performed between August 2006 and February 2008. Sixty-two patients in total were recruited. The mean total DAI score of patients enrolled was 9.8 ± 1.61 in the tacrolimus group and 9.1 ± 1.05 in the placebo group.

Drug Exposure

The mean trough concentrations in the tacrolimus group were 1.4 ± 0.9 ng/mL at 12 hours, 2.2 ± 1.5 ng/mL at 24 hours, 9.6 ± 3.1 ng/mL at day 7, 10.3 ± 3.1 ng/mL at day 8, 11.6 ± 3.4 ng/mL at day 10, and 13.0 ± 4.4 ng/mL at day 14.

Efficacy

Figure 1 shows that a clinical response was observed in 50.0% (16/32) of patients in the tacrolimus group and 13.3% (4/30) of patients in the placebo group. Significantly more patients in the tacrolimus group showed improvements compared with the placebo group ($P = 0.003$).

The observed rate of mucosal healing was 43.8% (14/32) in the tacrolimus group and 13.3% (4/30) in the placebo group ($P = 0.012$) at week 2, and clinical

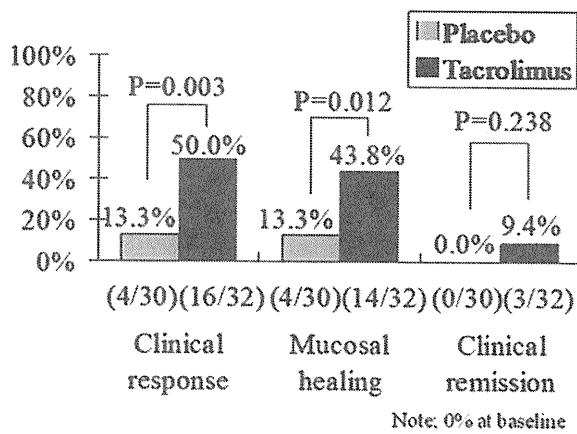


FIGURE 1. Efficacy result.

remission at week 2 was observed in 9.4% (3/32) of the tacrolimus group compared with 0.0% (0/30) in the placebo group ($P = 0.238$).

Twenty-seven of the 32 patients in the tacrolimus group achieved target trough levels. Among the 27 patients, the observed rate of clinical response, mucosal healing, and clinical remission were 59.3% (16/27), 51.9% (14/27), and 11.1% (3/27), respectively. Among the other five patients who did not achieve target trough levels, clinical response, mucosal healing, and clinical remission were not observed.

The rate of clinical remission was lower than that of mucosal healing. This was supposed to have been associated with the difference in criteria for the former and the latter. While mucosal healing was defined as achieving a mucosal appearance subscore of 0 or 1, clinical remission was more strictly defined as a subscore of 0 or 1 on each of the four factors (stool frequency, rectal bleeding, mucosal appearance, and physician's overall assessment) and a total score of 2 or lower.

Safety

Adverse events and serious adverse events were evaluated in all patients who received at least one dose of the study drug (Table 2). No statistically significant difference in incidence of adverse events was seen between the tacrolimus group (81.3%) and placebo group (70%) ($P = 0.379$).

The most common adverse event seen in patients who received tacrolimus was numbness. All events were mild and did not interfere with the patients' normal functioning. There were no significant adverse events on body temperature, blood pressure, pulse rate, hematologic parameters, electrolytes, renal function, cholesterol levels, and blood glucose levels, and no opportunistic infections were observed. No clinically significant differences in vital signs or laboratory test values were found between the two groups.

The mean values of serum creatinine (mg/dL) in the tacrolimus group and in the placebo group were, respec-

tively, 0.652 and 0.640 at baseline, and 0.633 and 0.672, respectively, at the end of the study. The mean values of BUN (mg/dL) in the tacrolimus group and in the placebo group were, respectively, 9.49 and 9.99 at baseline, and 11.59 and 9.29, respectively, at the end of the study.

Open-label Extension

After 2 weeks the treatment for 20 of the 62 patients in this study was changed to conventional treatment with drugs such as azathioprine. The remaining 42 patients continued to be treated with tacrolimus. Twenty-one of the 42 patients were in the tacrolimus group. The effect of continuous treatment in the tacrolimus group was evaluated by comparing the condition of 21 patients in the tacrolimus group at week 2 and week 12.

The results show an increase in mucosal healing from 66.7% (14/21) to 85.7% (18/21) and in clinical remission from 14.3% (3/21) to 28.6% (6/21) (Fig. 2a).

Seven of the 21 patients had failed azathioprine maintenance over the period beginning 3 months prior to the start of the study. Among the seven patients, the results also show an increase in mucosal healing from 71.4% (5/7) to 85.7% (6/7) and in clinical remission from 28.6% (2/7) to 57.1% (4/7). Among the other 14 patients the results also show an increase in mucosal healing from 64.3% (9/14) to 85.7% (12/14) and in clinical remission from 7.1% (1/14) to 14.3% (2/14).

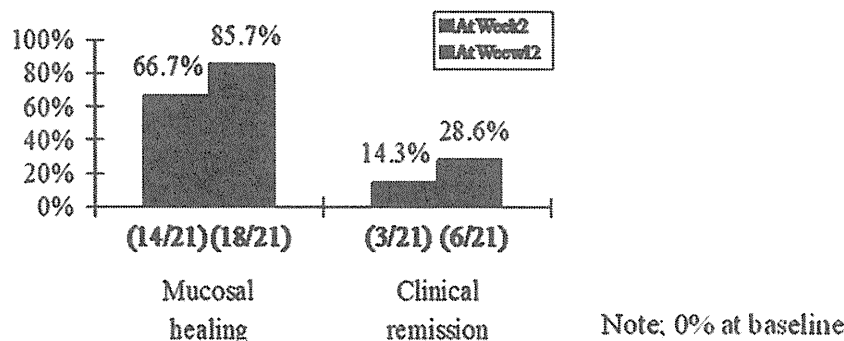
Furthermore, the mean prednisolone dose was decreased (8.9 mg/day) from that at baseline (24.2 mg/day) (Fig. 2b). One patient was off steroids at week 12 and the total DAI score of this patient was 3. Although the prednisolone doses was not evaluated after week 12, the prednisolone doses in six patients who achieved clinical remission

TABLE 2. Safety Result

No. of Patients (%)	Tacrolimus (n=32)	Placebo (n=30)
Adverse events	26 (81.3) ^a	21 (70.0)
Related adverse events	19 (59.4)	10 (33.3)
Serious adverse events:	None	None
Related adverse events occurring in > 5% of patients in at least one of the treatment groups		
Nausea	4 (12.5)	3 (10.0)
Headache	4 (12.5)	3 (10.0)
Numbness	4 (12.5)	0 (0.0)
Finger tremor	3 (9.4)	1 (3.3)
Dysmenorrhea	3 (9.4)	1 (3.3)
Hot flushes	2 (6.3)	1 (3.3)
Abdominal pain upper	2 (6.3)	1 (3.3)
Back pain	2 (6.3)	1 (3.3)

^aFisher's exact test, $P = 0.379$ vs. placebo.

a) Efficacy result of continuous treatment



b) Steroid tapering efficacy

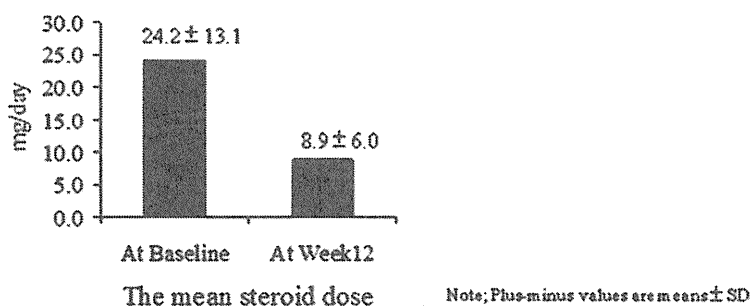


FIGURE 2. Open-label extension.

at week 12 were 10 mg/day, 10 mg/day, 5 mg/day, 5 mg/day, 2.5 mg/day, and 2.5 mg/day, respectively.

A smooth transition to the extension phase was achieved. The mean tacrolimus trough concentrations were 5.5 ± 1.5 ng/mL at week 4, 6.3 ± 1.7 ng/mL at week 8, and 6.7 ± 1.8 ng/mL at week 12.

This open-label extension phase of the study was well tolerated, with only minor side effects and no patients required colectomy.

Compliance

Patients were questioned by the investigator regarding compliance during the study. No cases of noncompliance could be identified.

DISCUSSION

Patients included in this study either had failed treatment with their most recent steroid treatment or were in immediate need of alternative treatment, including operative procedures. Because of these factors, a study design involving administration of placebo for 2 weeks or more was impossible both in terms of ethics and appropriate treatment. Although these results in the short duration of treatment should be treated with caution, it was demonstrated that oral tacrolimus therapy in patients with steroid-refractory,

moderate-to-severe UC shortened the acute phase and induced rapid mucosal healing.

An open-label extension resulted in further improvements and a reduction in steroid dose. Remission induction rates, relapse rates, and surgery rates in patients treated with tacrolimus over the long term are now being investigated in a prospective study.

The efficacy of tacrolimus in severe steroid-refractory UC was also confirmed in another small open-label study, although these results were not published. While intravenous infusion of cyclosporine has been thought to be effective and recognized as an alternative therapy against refractory, severe UC,^{9,10} administering oral tacrolimus therapy is more convenient than 24-hour continuous intravenous infusion of cyclosporine. Intravenous infusion imposes a great physical and psychological burden on the patient in hospital. Changing from intravenous injection to oral administration requires prolonged hospitalization to allow for the dose adjustment period; however, oral tacrolimus therapy can eliminate these disadvantages.

With regard to the long-term usefulness of tacrolimus, Baumgart et al¹¹ and Yamamoto et al¹² have reported the usefulness of long-term administration of tacrolimus for 12 weeks or more as remission maintenance therapy in open-label studies. More recently, Yamamoto et al¹³ reported the efficacy of tacrolimus compared with

thiopurines for maintaining remission in patients with refractory UC. They concluded that maintenance therapy with tacrolimus for patients with UC could be considered an alternative to thiopurine therapy.

Naganuma et al¹⁴ summarized how/when we should use tacrolimus in patients with refractory UC. Although our results suggest that tacrolimus therapy is useful as an alternative therapy against steroid-refractory UC, further investigation will be necessary to clarify the clinical usefulness of tacrolimus in comparison with biologics, such as infliximab, as a therapeutic strategy for refractory UC.

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Interval of Less Than 5 Years Between the First and Second Operation Is a Risk Factor for a Third Operation for Crohn's Disease

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Background: Previous studies have shown various risk factors for the initial and/or the second operation for Crohn's disease (CD). However, limited data are available with regard to the risk factors for a third operation. We aimed to clarify the risk factors for a third operation for CD.

Methods: A total of 200 CD patients who underwent a second intestinal surgery at 13 institutions were examined. We performed univariate and multivariate analyses to examine the influence of independent variables on the cumulative rate of needing a third operation.

Results: A total of 95 patients underwent a third operation. The overall 5-year and 10-year cumulative rates for the third operation were 42.2% and 71.0%, respectively. In univariate analysis, the interval between the initial and the second operation ($P = 0.0069$), postoperative administration of infliximab ($P = 0.0030$), and the anatomical site of the disease ($P = 0.0132$) were significant risk factors for the third operation. In multivariate analysis, the interval between the initial and the second operation ($P = 0.0287$) and postoperative administration of infliximab ($P = 0.0297$) remained significant risk factors for the third operation. The cumulative 5-year third operation rate was significantly higher in patients with an interval of less than 5 years between the first and second operations than for those with an interval of 5 years or more (47.8% versus 35.2%, $P = 0.0232$).

Conclusions: An interval of less than 5 years between the first and the second operations is a significant risk factor for a third operation in patients with CD.

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Key Words: Crohn's disease, surgery, reoperation, second surgery, risk factor, time trend, time changes

Approximately 50%–80% of patients with Crohn's disease (CD) require surgery at some point during their lifetime.^{1–3} Postoperative recurrence is common in CD, and after the initial operation some patients need a second and/

or multiple operations. Reoperation rates for recurrence range from 48%–71% at 20 years after the initial surgery.⁴ Furthermore, the risk of needing a third operation reaches 40% at 10 years after the second operation.^{5,6} Therefore, prevention of recurrence remains one of the major goals in the treatment of CD patients.

In order to prevent recurrence in CD, identification of patients at high risk for future recurrence is important because intensive therapy may be given to such patients to decrease recurrence needing surgical intervention. To identify such high-risk patients, previous studies evaluated various factors that potentially influenced the recurrence rates in CD patients, including age, gender, smoking, steroid use, duration of preoperative history, perforating disease, perianal disease, ileocolic disease, etc.^{1,2,7–13} However, these studies have focused on identifying risk factors for the initial or the second surgery. To date, few data have been generated with regard to the risk factors for the third operation except for one study with a comparatively small number of patients.¹⁴ Therefore, in the present study we aimed to evaluate risk factors for a third intestinal operation in a larger number of CD patients. We

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examined a total of 200 CD patients and showed that a shorter interval between the initial and the second operation was a significant risk factor for needing a third operation. To our knowledge, this is the first study that has shown that the interval between the initial operation and the second operation was a significant risk factor for a third operation. To the best of our knowledge, this is also the largest study of patients who underwent a second operation that has focused on the risk of a third intestinal operation for CD.

PATIENTS AND METHODS

Patients and Criteria for Diagnosis

A total of 200 CD patients who underwent initial and second intestinal surgeries were examined. Their onset of disease was between 1963–2003, and the diagnosis of CD was made according to the criteria provided by the Investigation and Research Committee for Intractable Inflammatory Bowel Disease organized by the Japanese Ministry of Public Welfare as described previously.¹⁵ The first and the second operation included intestinal surgery consisting of resection or strictureplasty. Surgeries for perianal disease or other minor surgical procedures without intestinal surgery were excluded from the initial and the second operations included in our study. This study was approved by the local Ethics Committee.

Data Management and Definitions

Case records were collected from 13 institutions which are participating in the Investigation and Research Committee for Intractable Inflammatory Bowel Disease organized by the Japanese Ministry of Public Welfare and scrutinized retrospectively. Data included the patient date of birth, date of onset of symptoms, date of diagnosis, disease localization at diagnosis, type of disease, type of surgery and date of initial/second surgery, and date of final follow-up, which were transferred to a data file (Microsoft Office Excel, Redmond, WA). The indications for surgery included acute abdominal pain, medical intractability, intestinal obstruction, palpable mass/abscess, internal fistulas, colonic dilatation, etc. The disease localization was established at the time of diagnosis and was classified into three groups: 1) small bowel disease (inflammation of the small bowel); 2) ileocolic disease (inflammation involving both the small bowel and the colon); 3) colorectal disease (inflammation confined to the colon or rectum or both). The type of disease was classified into perforating or nonperforating disease, as described previously.¹¹ Perforating disease included patients who underwent their first operation due to perforating disease, whereas nonperforating disease patients were those who underwent the initial operation due to another cause, such as intestinal obstruction, medical intractability, hemorrhage, etc. Perforating disease was classified as perforating, regardless of the concomitant presence of additional nonperforating disease. The primary outcome measure of this study was the rate of patients needing a third intestinal resection or strictureplasty.

Statistical Analysis

The statistical analysis was performed using the JMP software program (SAS Institute, Cary, NC). The cumulative third operation rate was calculated by the Kaplan–Meier method and compared by log-rank test. Univariate and multivariate analyses were performed by Cox proportional hazards regression models in order to examine the influence of independent variables on the cumulative probability of the third operation. Variables with $P < 0.1$ in univariate analysis were entered into each multivariate analysis. $P \leq 0.05$ was considered statistically significant in all analyses. Probability values and confidence intervals were calculated at the 95% level.

RESULTS

Patient Characteristics

Table 1 shows the characteristics of patients. In the 200 CD patients who underwent a first and second intestinal operation, 95 patients underwent a third intestinal surgery after a median of 3.5 years. The frequency of ileocolic disease or administration of infliximab was significantly higher in patients who underwent the third operation than for those who did not. The overall 5-year and 10-year cumulative rates of needing a third operation were 42.2% and 71.0%, respectively (Fig. 1).

Risk Factors for Reoperation and Cumulative Rate of Reoperation

The impact of possible risk factors that may have influenced the frequency of the third operation was evaluated by univariate and multivariate analyses (Table 2). In an analysis of duration of disease, we evaluated the following three different types of disease duration with respect to the risk of a third operation: first, the period between disease onset and the first operation; second, the period between disease onset and the second operation; and last, the interval between the first and the second operation. In a univariate analysis, significant risk factors for the third operation were the interval between the first and the second operation, the anatomical site of the disease, and postoperative administration of infliximab. The cumulative risk of the third operation was significantly higher in patients whose interval between the first and second operations was less than the median interval (4.7 years). We next examined whether the same trend could be observed when we divided patients according to the interval of either shorter or longer than 5 years between the surgeries. Patients whose interval between the initial and the second operation was 5 years or less also showed a higher risk of requiring a third operation (hazard ratio = 0.617 (95% confidence interval [CI], 0.401–0.935, $P = 0.0226$) compared to the patients whose interval was longer than 5 years. With regard to the anatomical site of the disease, patients with ileocolic disease showed significantly higher risk of

TABLE 1. Patient Characteristics

	Patients Who Received a 3rd operation (n = 95)	Patients Who Underwent Only 2nd operations (n = 105)	P-value	Total Number of Patients (n = 200)
Age at diagnosis				
Median	21.1	22.6	<i>P</i> = 0.439	22.4
(Range)	(8–49)	(10–53)		(8–53)
Gender			<i>P</i> = 0.9417	
Male	71	78		149
Female	24	27		51
Disease location			<i>P</i> = 0.0006	
Ileocolic	69	56		125
Small bowel	26	38		64
Colorectal	0	11		11
Type of disease			<i>P</i> = 0.3032	
Non-perforating	43	59		102
Perforating	45	40		85
Others	7	6		13
Postoperative medication				
Immunosuppressants			<i>P</i> = 0.6132	
+	17	16		106
–	78	89		94
Infliximab			<i>P</i> = 0.0170	
+	30	13		48
–	65	87		152
Elemental diet			<i>P</i> = 0.6175	
+	56	63		119
–	39	41		80
Others	0	1		1
Disease duration between onset and the first surgery (yrs)				
Median	3.6	4.0	<i>P</i> = 0.1494	4.0
(Range)	(0–22.4)	(0–19.8)		(0–22.4)
Disease duration between onset and the second operation (yrs)				
Median	7.8	11.3	<i>P</i> = 0.0101	9.8
(Range)	(1.0–27.0)	(1.0–43.2)		(1.0–43.2)
Interval between the first and the second operations (yrs)				
Median	4.0	5.1	<i>P</i> = 0.0897	4.7
(Range)	(0.1–26.0)	(0.5–43.2)		(0.1–43.2)

Duration before surgery: duration between diagnosis and the first surgery.
 Reoperation: reoperation after the first surgery.

needing a third operation than patients with either colorectal-only disease or small intestine disease. Other factors such as gender, age at diagnosis, preoperative duration of disease, and type of disease did not show any significant correlation with the third operation rate.

Next, we performed a multivariate analysis among the three risk factors that showed a significant impact on the rate of requiring a third operation by univariate analysis (Table 2). In multivariate analysis, the interval between the

first and the second operation, and the use of infliximab remained significant risk factors.

Cumulative Rate of Patients Requiring a Third Operation

Cumulative 5-year and 10-year rates of the need for a third operation were significantly higher in patients whose interval between the first and the second operation was 4.7 years or less (*P* = 0.0069) (Fig. 2). Also patients

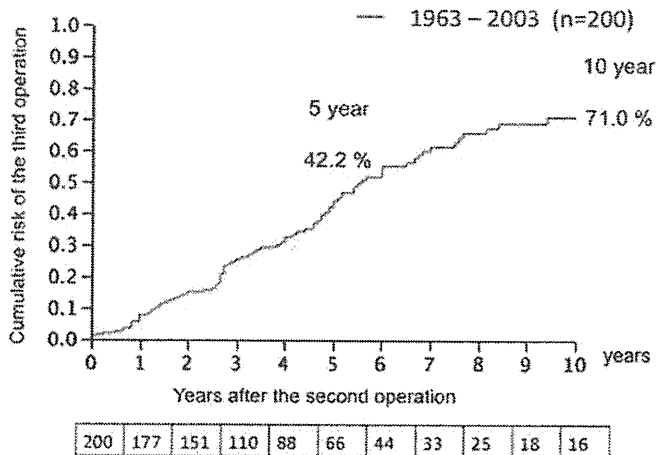


FIGURE 1. The overall 5-year and 10-year cumulative rate of third operations for CD in Japan. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

whose interval was 5.0 years or less showed a higher third operation rate than those with interval more than 5 years (5-year third operation rate; 47.8% versus 35.2%, $P = 0.0232$). Cumulative 5-year and 10-year rates of the need for a third operation were also significantly higher in patients who received infliximab postoperatively than those who did not ($P = 0.0015$) (Fig. 3). With regard to the disease localization, patients with ileocolic disease showed a significantly higher rate of needing a third operation than those with small bowel or colonic disease ($P = 0.0154$) (Fig. 4).

DISCUSSION

The present study showed that the disease interval between the first and the second operation was a significant risk factor for CD patients to need a third operation. A shorter interval was significantly associated with a higher risk of needing a third operation. The 5-year cumulative rate of patients needing the third operation was 47.8% in those with an interval between the first and second surgeries of 5 years or less, while only 35.2% in patients whose interval was more than 5 years ($P = 0.0232$). These results suggest that intensive postoperative adjuvant therapy may be especially important for CD patients who have an interval of 5 years or less between the initial and second surgeries in order to avoid the need for a third operation.

Previous studies demonstrated various risk factors for intestinal operation in CD, however, most of these factors have been focused on predicting the risk of needing the first or second operation.^{1,7-13} The risk factors for needing a third operation were unclear. To our knowledge, there has been only one study by Alves et al¹⁴ that evaluated risk factors for a third operation in CD. However, in Alves et al's study, the total number of patients was comparatively small. In their study, there were 28 CD patients who received a sec-

ond intestinal operation, but since two cases were excluded because of missing data, they only examined a total of 26 CD patients. In the present study, we examined 200 CD patients who underwent a second operation, and to our knowledge, this is the largest number of patients among studies evaluating the risk of needing a third operation for CD. Another difference between Alves et al's study and the present study is the data source. Alves et al's study was based on the data from a single institution. However, a single institution-based study cannot rule out the possibility of patient selection biases. On the other hand, in the present study, we collected data from 13 institutions in Japan and we were able to analyze the risk factors for the third operation based on the multiinstitutional dataset.

Alves et al¹⁴ showed that the third intestinal resection rate was significantly lower in patients treated with immunosuppressive drugs (azathioprine and 6-mercaptopurine, or methotrexate) than in untreated patients (17% versus 58%, $P < 0.02$). However, with regard to the postoperative effect of immunosuppressants in preventing recurrence, previous studies have shown conflicting results.¹⁶⁻²² For example, Hanauer et al and D'Haens et al^{16,18} showed that the postoperative recurrence rate was significantly lower in patients receiving immunosuppressants than in those receiving placebo. A meta-analysis also showed that immunosuppressants are more effective than placebo in preventing both clinical and endoscopic postoperative recurrence in CD.²⁰ On the other hand, Ardizzone et al¹⁷ reported that there was no difference in the efficacy of immunosuppressants in preventing clinical and surgical relapses after conservative surgery. In the present study, administration of immunosuppressants was not a significant risk factor for needing a third operation. On the other hand, postoperative administration of infliximab was a significant risk factor for the patients needing a third operation in the present study. This is contrary to the results of recent studies, which showed that infliximab is effective for reducing the postoperative recurrence rate.²³⁻²⁷ In a recent randomized controlled study, Regueiro et al²⁵ showed that endoscopic (9.1% versus 84.6%, $P = 0.0006$) and histologic (27.3% versus 84.6%, $P = 0.01$) recurrence rates were significantly lower in CD patients who received infliximab after intestinal resective surgery compared to patients who received placebo. One reason for the conflicting results between the present and other studies may be due to a selection bias of the patients who received infliximab. In the present study, patients received infliximab for therapy of recurrent disease. Therefore, there is a possibility that infliximab might have been administered preferably to higher-risk patients for a third operation, while lower-risk patients did not receive these treatments. This patient selection bias may have been responsible for the results indicating infliximab to be a risk factor in the present study.

TABLE 2. Results of Univariate and Multivariate Analyses

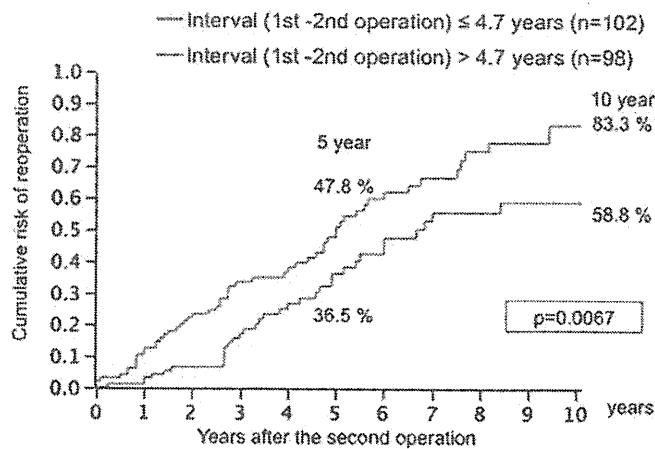
	Univariate analysis			Multivariate Analysis		
	Hazard Ratio	95% CI	P-value	Hazard Ratio	95% CI	P-value
Age at diagnosis						
≤22	0.862	0.571–1.295	<i>P</i> = 0.4747			
>22						
Gender						
Male	0.772	0.474–1.214	<i>P</i> = 0.2686			
Female						
Disease location						
Small bowel, Colorectal	1.749	1.128–2.799	<i>P</i> = 0.0132	1.498	0.946–2.413	<i>P</i> = 0.0859
Ileocolic						
Type of disease						
Perforating	1.375	0.903–2.098	<i>P</i> = 0.1370			
Nonperforating						
Postoperative medication						
Immunosuppressants						
+	1.321	0.752–2.191	<i>P</i> = 0.3188			
–						
Infliximab						
+	1.999	1.274–3.068	<i>P</i> = 0.0030	1.676	1.054–2.614	<i>P</i> = 0.0297
–						
Elemental diet						
+	0.907	0.603–1.377	<i>P</i> = 0.6413			
–						
Disease interval between the first and the second operation						
≤4.7	0.566	0.371–0.856	<i>P</i> = 0.0069	0.626	0.407–0.953	<i>P</i> = 0.0287
>4.7						
Disease duration before the first operation						
≤4.0	1.047	0.691–1.575	<i>P</i> = 0.8263			
>4.0						
Disease duration before the second operation						
≤9.8	0.693	0.454–1.045	<i>P</i> = 0.0802			
>9.8						

Duration before surgery: duration between diagnosis and the first surgery.
Reoperation: reoperation after the first surgery.

The second reason may be a shorter follow-up period for patients who received infliximab. This is actually one limitation of the present study, because we were unable to evaluate the effect of infliximab with a long enough follow-up period because infliximab did not become available in Japan until 2002. In the present study, more than half of the patients (110 patients) underwent the third operation in or after 2002. Among these patients, the median follow-up period was only 2.8 years. We believe we need to follow patients for a longer period of time to evaluate the true effect of infliximab in the adjuvant setting.

The present study showed that an interval of less than 5 years between the first and the second operation for

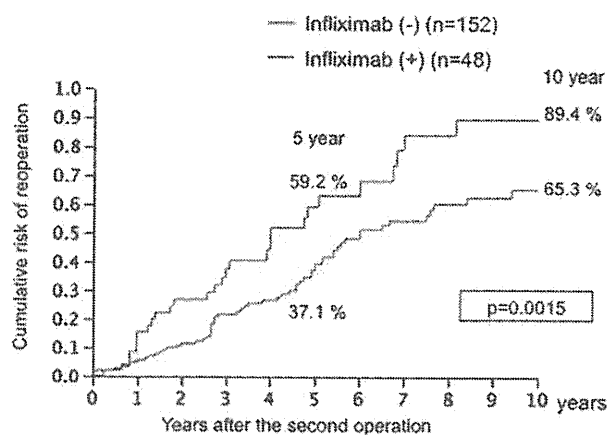
CD was a significant risk factor for needing a third operation. Previous studies have also shown that there is a correlation between the duration of the disease and a risk of surgery. A number of studies have shown a higher risk of surgery with a shorter history of disease.^{28–30} However, these studies examined the relationship between the disease duration before the first operation and this risk of a second operation. None of the previous studies examined the possible role of disease interval on the risk for needing a third operation. We have shown that patients who underwent a second surgery within 5 years of the first operation are at a higher risk of needing a third operation. With regard to the interval between operations, Greenstein et al¹¹ examined



≤4.7	98	85	72	50	42	33	20	14	10	6	4
>4.7	102	92	79	60	46	33	24	19	15	12	12

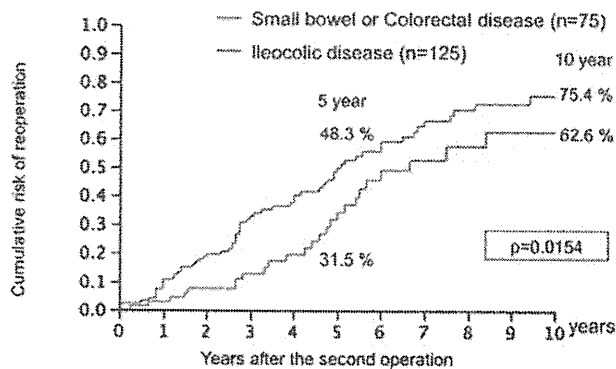
FIGURE 2. The cumulative rate of third operations depends on the interval between the first and the second operations. The cumulative risk of the third operation was significantly higher in patients whose interval between the first and second operations was less than 4.7 years ($P = 0.0067$).

770 patients with CD and reported that third operations occurred faster after second operations than did second operations after the first. Also, in an analysis of CD patients who had undergone multiple operations, Greenstein et al³¹ showed that as patients undergo repeated surgical procedures, their postoperative recurrences develop faster after each successive operation. In the present study the mean interval between the second and the third operation (4.4 years) was significantly shorter than that between the



Infliximab (-)	152	137	119	86	72	54	36	28	21	15	13
Infliximab (+)	48	41	33	24	16	12	8	5	3	3	3

FIGURE 3. The cumulative rate of third operations depends on the postoperative administration of infliximab. The cumulative risk of the third operation was significantly higher in patients who received postoperative infliximab ($P = 0.0015$).



Small bowel or Colorectal disease	74	68	59	46	37	28	17	14	9	9	9
Ileocolic disease	125	109	92	64	51	38	27	19	16	11	9

FIGURE 4. The cumulative rate of third operations depends on the localization of the disease. The cumulative risk of the third operation was significantly higher in patients with ileocolic disease ($P = 0.0154$).

first and the second operation (5.8 years) ($P = 0.019$). Our results were in accordance with Greenstein et al's observations.

To our knowledge, the present study was the largest study of CD patients after the second surgery showing the risk of needing a third surgery. These results suggest that patients with a short interval between the first and the second operations need intensive adjuvant therapy postoperatively, such as infliximab. On the other hand, in the present study the disease duration before the first operation was not a significant risk factor for needing a third operation.

Another risk factor for the third operation in the present study was the anatomical site of the disease. Patients who have colorectal involvement will often require a definitive resection with a permanent stoma. In fact, none of the patients with colonic-only disease underwent a third operation in our study. Therefore, we examined patients with small intestine disease and colonic-only disease together against patients with ileocolic disease. By univariate analysis we showed that ileocolic disease was a significantly higher risk factor for needing a third operation, although this did not remain significant by multivariate analysis.

Previously, a number of studies examined the impact of anatomical site of disease on the recurrence, and many studies have demonstrated that the risk of recurrence was highest for ileocolic disease and lowest for colonic-only disease.³²⁻³⁵ However, most of these studies examined the impact of the anatomical site on the first and/or the second surgery, and therefore, data concerning the need for a third operation with regard to the anatomical site involved is limited. In agreement with previous studies of initial and second surgeries, the present study indicated that there is a significantly higher risk of needing a third operation in

patients with ileocolic disease. The present study shows ileocolic disease to be a risk factor, not only for the first or the second operation, but also for the third operation for CD.

Another unique factor that might affect the risk of needing surgery in Japanese CD patients is the use of the elemental diet (ED) therapy. In Japan, ED, rather than corticosteroid therapy, is considered to be effective in the primary remission-induction therapy for active intestinal inflammation.^{36–38} A Japanese randomized controlled trial showed that a “half elemental diet” therapy regimen, in which half of the daily calorie requirement is provided by an elemental diet and the remaining half by a free diet, is effective in reducing the relapse rate compared with patients eating purely a free diet (relapse rate: 34.6% versus 64.0%).³⁶ However, in the present study ED was not a significant factor for needing a third operation.

One of the limitations of the present study is that we could not examine the association between the third operation and several well-established risk factors including smoking, steroid use, and perianal disease, since they were not available in retrospective review.^{7,9,10,12,13} Although these factors are known to be associated with the operation rate, it still remains unclear how these factors affect the risk of the third operation. We believe further studies are necessary to clarify this point. Another important issue is the endpoint of the present study. In the present study we included both intestinal resections and strictureplasties as an intestinal operation. Ideally these two procedures need to be analyzed separately. However, some patients receive both intestinal resections and strictureplasties at the same time, and furthermore the number of each procedure differs between each individual. Therefore, in the present study we did not divide patients according to each procedure. However, we believe that each procedure as well as the number of procedures needs to be evaluated separately. Lastly, although we examined multiple factors in association with the risk of third operation, the number of patients was comparatively small. Therefore, in order to clarify these issues we believe that a prospective study with a large number of patients is necessary.

In conclusion, to our knowledge, the present study is the first to show that a shorter interval between the first and the second operations is a significant risk factor for needing a third operation. Patients whose interval between initial and second surgeries is 5 years or less are at a higher risk of recurrence and, therefore, should receive postoperative adjuvant therapy to prevent the need for a third operation. However, to further confirm this we need to prospectively evaluate CD patients with a longer follow-up period. This is particularly important because the use of infliximab, which is generally thought to reduce disease symptoms and recurrence, was a risk factor for the third operation in our study.

However, due to the retrospective nature of the present study, this was considered to be due to a bias, because these drugs might have been administered more frequently to higher-risk patients. Nevertheless, further studies are needed to confirm whether this is indeed the case.

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