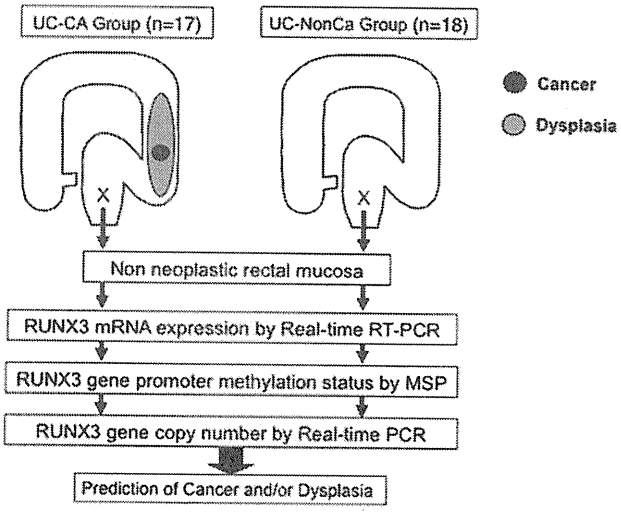

Figure 1. Sample collection and data analysis procedure. Samples were obtained from non-neoplastic rectal mucosa for RNA and DNA extraction. *RUNX3* mRNA expression level, hypermethylation of *RUNX3* promotor region and *RUNX3* DNA copy number were compared between UC-Ca and UC-NonCa groups.

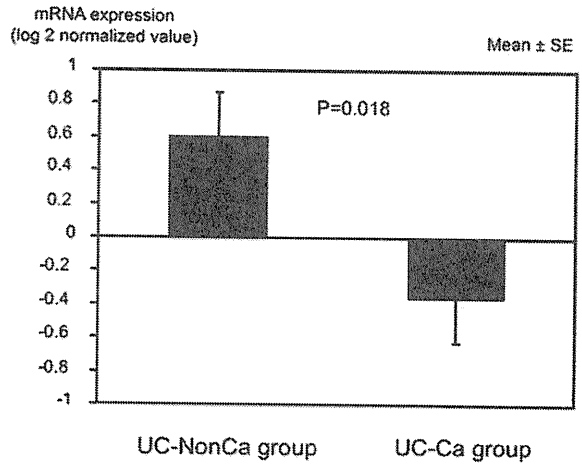

Figure 2. Mean mRNA expression of *RUNX3*. UC-NonCa group showed significantly higher *RUNX3* mRNA expression level than UC-Ca group (p=0.018). Vertical axis shows normalized *RUNX3* mRNA expression level. (A bar indicates standard deviation).

Table II. Methylation status of RUNX3 gene promoter region.

	No. of cases with hypermethylation	No. of cases without hypermethylation	Frequency of hypermethylation (%)
UC-Ca group (n=17)	0	17	0
UC-NonCa group (n=18)	0	18	0

calculated according to expression level or copy number for the ability of the logistic regression models to differentiate between UC-Ca and UC-NonCa patients. The predictive accuracy was determined by measuring the specificity, sensitivity, and area under the ROC curve. A predictive model with an ROC of ≥0.7 is considered to have good discrimination, and an area under the ROC curve of 0.5 is equivalent to a 'coin toss'.

Statistical analysis. Associations between *RUNX3* gene expression, promoter methylation, DNA copy number and clinical outcomes were tested using unpaired t-tests, generalized logistic model analysis or Fisher's exact test, as appropriate. Data were analyzed using JMP ver 5.0 software (SAS Institute, Tokyo).

Results

mRNA expression level by RT-PCR analysis. Gene expression levels of RUNX3 were significantly lower (1.9-fold) in the UC-Ca group compared with the UC-NonCa group (p=0.018) (Fig. 2). Mean Log2-normalized mRNA expression level for UC-Ca group and UC-NonCa group was -0.35 and 0.6, respectively.

Methylation status of RUNX3 gene promoter region. There was no significant difference in the methylation status of the *RUNX3* gene promoter region, with neither group showing hypermethylation (Table II).

DNA copy number of RUNX3. The mean copy number of *RUNX3* was significantly greater in the UC-NonCa group compared with the UC-Ca group (3.04 vs. 1.99, p=0.0016) (Fig. 3).

mRNA expression level and DNA copy number of RUNX3. mRNA expression levels were then examined according to DNA copy number. The standard deviation of the *RUNX3* copy number of non-neoplastic tissue adjacent to sporadic CRC tumours was 0.1 (data not shown), so we determined the cut-off value for copy number gain as 2.2 (2.0 + 2 x SD). Samples were divided into a high copy number (>2.2) or low copy number (≤2.2) group. The *RUNX3* mRNA level was significantly higher in the high copy number group compared

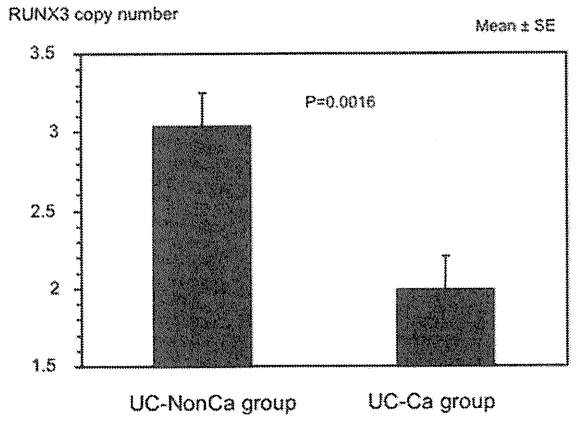

Figure 3. *RUNX3* DNA copy number. UC-NonCa group showed significantly higher *RUNX3* DNA copy number than UC-Ca group (3.04 vs. 1.99, p=0.0016).

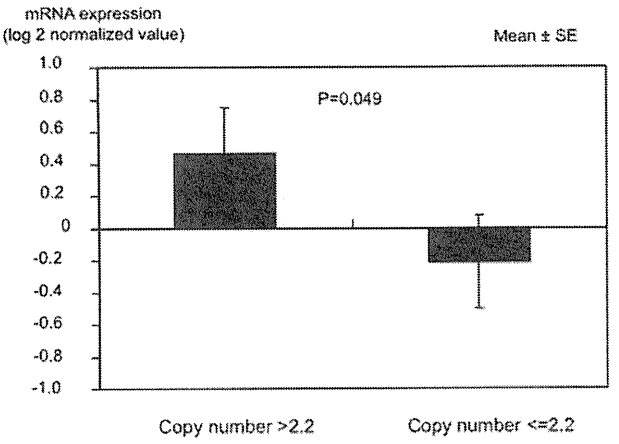

Figure 4. *RUNX3* mRNA expression level and DNA copy number. High copy number group (>2.2) showed significantly higher mRNA expression level than low copy number group (≤2.2) (p=0.049).

to the low copy number group (p=0.049) (Fig. 4). Mean Log2-normalized mRNA expression level for the high copy number group and the low copy number group was 0.47 and -0.21, respectively.

Prediction of UC-associated neoplasm development by RUNX3 mRNA level and DNA copy number. ROC analysis against UC-associated neoplasms revealed the AUC to be 0.74 and the copy number to be 0.85 (Fig. 5). To maximize sensitivity and specificity, we set the cut-off points as 0.027 and 2.06 for mRNA and DNA copy number, respectively. Using mRNA expression data, we were able to predict the development of UC-associated neoplasms with an accuracy of 74.3%. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were 76.5%, 72.2%, 72.2% and 76.5%, respectively. There were nine misclassifiers: five in the UC-Ca group and four in the UC-NonCa group. Using

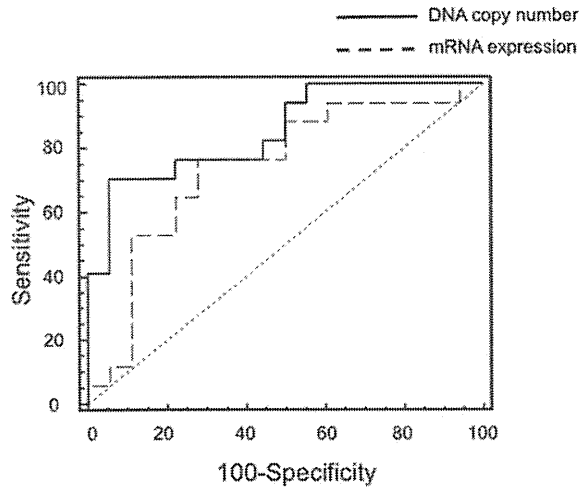

Figure 5. ROC curve for UC-Cancer prediction model based on *RUNX3* mRNA expression and DNA copy number. Predicted probability of UC-associated cancer computed from logistic regression models including *RUNX3* mRNA and DNA copy number. Specificity and sensitivity computed at each possible cut-off on predicted probability for two models. AUC values compared for two models.

DNA copy number data, we were able to predict the development of UC-associated neoplasms with an accuracy of 82.9%. The sensitivity, specificity, PPV and NPV were 70.6%, 94.4%, 92.3% and 77.3%, respectively. There were six misclassifiers: one in the UC-Ca group and five in the UC-NonCa group.

Discussion

In the current analysis of *RUNX3* expression in non-neoplastic rectal mucosa of UC patients, we were able to show that non-UC-Ca patients have significantly higher *RUNX3* mRNA expression levels than UC-Ca patients. Increased DNA copy numbers of *RUNX3* are a plausible mechanism for this change. Based on the DNA copy number, we were able to predict the development of UC-associated neoplasms with an accuracy of 82.9%. The present model may enable more efficacious surveillance for UC patients by selecting high risk UC-Ca patients.

In UC-carcinogenesis, it is suggested that there is a field effect of chronic inflammation with a need for constantly increased re-epithelialisation and the suppression of cell cycle-regulating proteins, which may make genes susceptible to genetic damage. Indeed, previous studies have shown that specific gene alteration is already present in the non-neoplastic mucosa of UC-CA patients (5-8). However, to date, *RUNX3* has not been identified as one of these genes. This is the first study to show altered *RUNX3* expression levels in UC-Ca patients. *RUNX3* expression is decreased in various cancers, (10-12) and was significantly decreased in UC-Ca patients compared with UC-NonCa patients in the present study. From these results, it is difficult to conclude that there is a causal relationship between *RUNX3* expression levels and the development of UC-Ca because there is no concrete evidence to support this hypothesis. However, considering that RUNX3

is a tumor suppressor gene, decreased expression of *RUNX3* may help to increase the risk of developing UC-Ca in long-standing UC patients.

Next, we examined the mechanisms of increased *RUNX3* expression in UC-NonCa patients. Previous studies have shown that hypermethylation plays an important role in controlling *RUNX3* mRNA expression levels in various cancers including gastric cancer (13-19). Therefore, we first examined the hypermethylation of CpG islands in the promotor region of *RUNX3*. However, we failed to show any relation between hypermethylation status and *RUNX3* expression level because not a single case in either UC-Ca or UC-NonCa group showed the hypermethylation in *RUNX3* promoter DNA.

Recently, DNA copy number has gained attention as a possible mechanism for altering gene expression in various diseases (27,28). Copy number variations may be either inherited or caused by *de novo* mutations and can influence gene expression (28,29). In inflammatory bowel disease, changes in the DNA copy number have been reported in Crohn's disease. Fellermann *et al* reported that changes in the human ß-defensin 2 gene copy number at the ß-defensin locus predisposes individuals to Crohn's disease, most likely through diminished ß-defensin expression (27). We therefore focused on DNA copy number as a possible regulator of *RUNX3* mRNA expression and found that the UC-NonCa group had both higher copy numbers and *RUNX3* expression levels than the UC-Ca group. These results suggest that increased expression of *RUNX3* mRNA in UC-NonCa patients might be attributable to the increased *RUNX3* DNA copy number. It is not known when the copy number changes occurred in UC-NonCa patients, but considering that the risk of UC-Ca increases with the duration of disease, we speculate that copy number changes might be acquired as *de novo* mutations during the long period of disease duration.

An important clinical implication of the present study is the prediction of patients at a higher risk of developing UC-Ca. In predicting the development of UC-associated neoplasm, we used mRNA expression level and DNA copy number. Although ROC analysis showed a higher AUC for DNA copy number (0.85) than mRNA expression level (0.74), the difference did not reach statistical significance and seemed to be attributable to a comparatively small number of cases. In fact, the overall predictive accuracy was higher by DNA copy number (82.9%) than mRNA expression level (74.3%), suggesting that RUNX3 DNA copy number is a more efficacious predictive marker than mRNA expression level. The accuracy level in the present model was not as high as our previous DNA microarray study, which showed a 98.1% accuracy rate, but required 40 genes to achieve this prediction accuracy. On the other hand, the present study only assessed *RUNX3* DNA copy number, so is a more cost-effective method and is easier to apply in clinical settings.

One limitation of the present study is that because of a comparatively small number of patients, we could not validate the accuracy of the predictive model. To further confirm its accuracy, the *RUNX3* DNA copy number should be validated by testing at another institution, preferably with a prospective study of a larger number of patients. The second point is that *RUNX3* expression levels are influenced by the degree of inflammation (20) which cannot be excluded from influencing the current results. However, in the present study, UC patients with active inflammation were excluded from analysis. Finally, the precise mechanism of action of *RUNX3* has not been fully clarified, and analysis of this would greatly contribute to our understanding of the mechanisms of UC-Ca.

In conclusion, patients without UC-Ca showed higher expression of *RUNX3* than those with UC-Ca. We show that this increase could have been caused by an increase in *RUNX3* DNA copy number. Furthermore, using the *RUNX3* copy number, we were able to predict patients with and without UC-Ca with an accuracy of 80%, and suggest that this predictive model may be useful in detecting patients at high risk of developing UC-Ca, thereby increasing the efficacy of surveillance colonoscopy for UC patients.

Acknowledgements

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Original Study

Predicting Ulcerative Colitis-Associated Colorectal Cancer Using Reverse-Transcription Polymerase Chain Reaction Analysis

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Abstract

Background: Widespread genetic alterations are present not only in ulcerative colitis (UC)-associated neoplastic lesions but also in the adjacent normal colonic mucosa. This suggests that genetic changes in nonneoplastic mucosa might be effective markers for predicting the development of UC-associated cancer (UC-Ca). This study aimed to build a predictive model for the development of UC-Ca based on gene expression levels measured by reverse-transcription polymerase chain reaction (RT-PCR) analysis in nonneoplastic rectal mucosa. **Patients and Methods:** Fifty-three UC patients were examined, of which 10 had UC-Ca and 43 did not (UC-NonCa). In addition to the 40 genes and transcripts previously shown to be predictive for developing UC-Ca in our microarray studies, 149 new genes, reported to be important in carcinogenesis, were selected for low density array (LDA) analysis. The expression of a total of 189 genes was examined by RT-PCR in nonneoplastic rectal mucosa. **Results:** We identified 20 genes showing differential expression in UC-Ca and UC-NonCa patients, including cancer-related genes such as *CYP27B1*, *RUNX3*, *SAMSN1*, *EDIL3*, *NOL3*, *CXCL9*, *ITGB2*, and *LYN*. Using these 20 genes, we were able to build a predictive model that distinguished patients with and without UC-Ca with a high accuracy rate of 83% and a negative predictive value of 100%. **Conclusion:** This predictive model suggests that it is possible to identify UC patients at a high risk of developing cancer. These results have important implications for improving the efficacy of surveillance by colonoscopy and suggest directions for future research into the molecular mechanisms of UC-associated cancer.

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Keywords: Dysplasia, Prediction, Neoplasia, Inflammatory bowel disease, RUNX3

Introduction

Colon cancer is a well known complication of ulcerative colitis (UC) and the cumulative risk of developing UC-associated colon cancer increases with the duration and extent of the disease.[1-3] Therefore, patients with total colitis, whose disease has lasted over 7 years, are considered to be at high risk of developing cancer and are recommended to undergo regular colonoscopy.[1-3] However, to improve the efficacy of surveillance, and provide more selective treatment strategies, more effective markers for identifying patients at a higher risk of developing cancer are urgently needed.

UC-associated colon cancer develops in a different way from sporadic colon cancer, and is sometimes called an "inflammation

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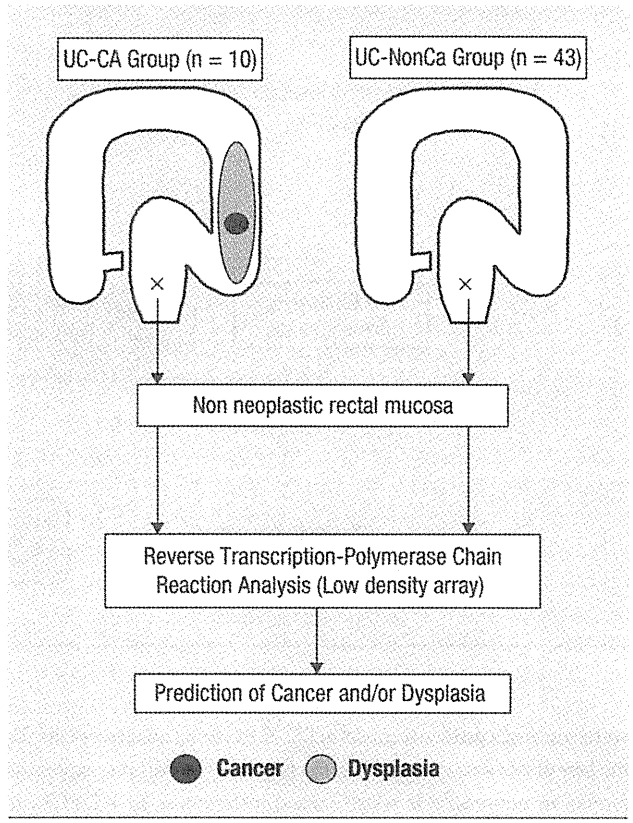
Table 1 Characteristics of Patients in the 2 Groups

Characteristic	UC-Ca Group (n = 10)	UC-NonCa Group (n = 43)
Age at Biopsy, Years		
Mean (Range)	51.9 (36-76)	47.1 (22-69)
SD	15.3	15.3
Age at UC Diagnosis, Years		
Mean (Range)	35.9 (21-55)	32.6 (17-52)
SD	13.4	10.3
Sex, n		
Male	7	27
Female	3	16
Duration of Disease, Years		
Mean (Range)	16.0 (7-27)	14.5 (7-30)
SD	6.2	6.9
Extent of Disease, n		
Total Colitis	10	43
Left-Sided Colitis	0	0
Proctosigmoiditis	0	0
Proctitis	0	0
Degree of Inflammation, n		
None	3	11
Mild	4	20
Moderate	3	11
Severe	0	1
Medication, n		
Mesalamine	8	38
Corticosteroids	1	5
6mp/Aza	0	0
CSA	0	0
PSC, n		
Present	0	0
Absent	10	43

P values for all characteristics group comparisons were not significant.
Abbreviations: 6MP/AZA = 6-mercaptopurine/azathioprine; Ca = cancer; CSA = cyclosporine A; PSC = primary sclerosing cholangitis; UC = ulcerative colitis.

Figure 1 Flow Chart Illustrating Sample Collection and Data Analysis Procedures. Samples were obtained from nonneoplastic rectal mucosa of patients with ulcerative colitis for RNA extraction. Gene expression was determined using TaqMan low density arrays based on real-time polymerase chain reaction (PCR) and used to carry out class prediction

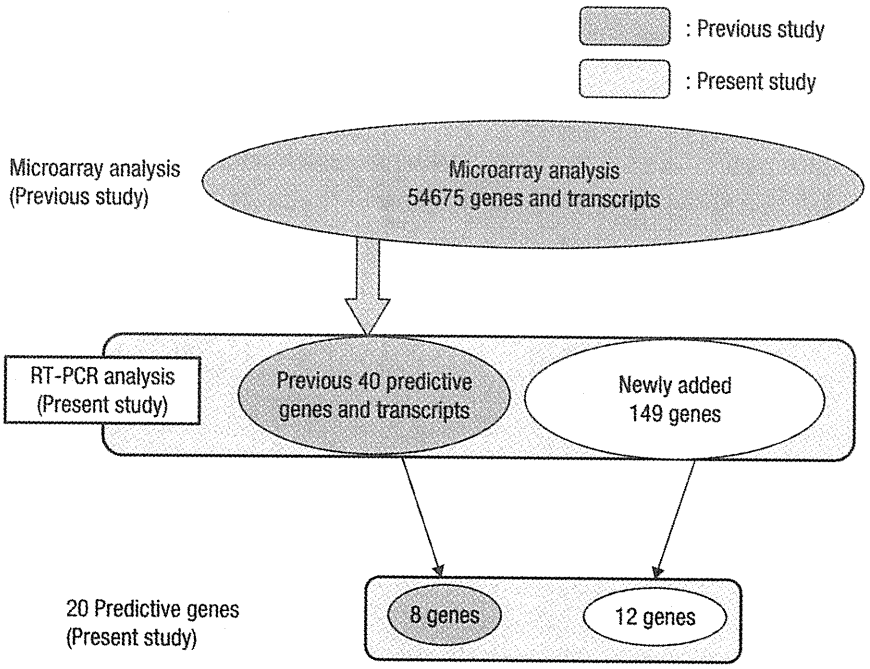


dysplasia carcinoma sequence."[4-6] Dysplasia is often seen in patients with UC-associated cancer around or at a distance from cancer lesions and it is considered to be a precursor of cancer.[1] It has also been suggested that there are some differences in the genetic changes in sporadic and UC-associated colon cancers. Previous studies have shown that patients with UC-associated colon cancer have widespread genetic alterations not only in neoplastic lesions but also in the nonneoplastic colonic mucosa, referred to as a "field effect."[7-12] These studies suggested that genetic changes in the nonneoplastic mucosa might be effective markers for predicting the development of UC-associated colon cancer.

We previously performed DNA microarray analysis of the nonneoplastic colonic mucosa of UC patients and were able to build a predictive model for the development of UC-associated colon cancer.[13] However, microarray analysis is known to lack reproducibility as a means of analyzing gene expression quantitatively and it requires verification using an alternative quantitative method. This constraint still limits the use of microarrays in clinical practice. However, real time reverse transcription polymerase chain reaction (RT-PCR) is a more reliable method for quantifying gene expression and it is widely used to validate the expression levels of genes identified in microarray analyses.[14-18] Recently, RT-PCR analysis has been used in the prediction of outcomes in a variety of diseases. For example, studies have shown that RT-PCR analyses of selected genes can accurately predict outcomes for patients with diffuse large–B-cell lymphoma and lung cancer.[15,16]

Therefore, in this study, we aimed to clarify how well RT-PCR analysis could be used to predict the development of UC-associated colorectal cancer (UC-CRC). In addition to 40 genes and transcripts that we previously used in a predictive model, based on our microarray study, we selected an additional 149 genes that have been shown to be important in the development of cancer in various organs including the colon and rectum. We validated levels of expression of these genes by RT-PCR and

Figure 2 Study Design. In addition to the 40 discriminator genes and transcripts used in our previous study, 149 new genes that have been reported to be important in carcinogenesis were used in the low density array (LDA) analysis. Finally 20 genes were selected as predictor genes



we formulated a predictive model of UC-CRC based on these results. To the best of our knowledge, this is the first study to use gene expression profiles in nonneoplastic rectal mucosa, determined by RT-PCR, to predict the development of UC-CRC.

Materials and Methods

Patients and Samples

This study used the same set of 53 UC patients as our previous microarray study[13] and their characteristics are shown in Table 1. Informed consent was obtained from all patients for the collection of specimens, and the study protocol was approved by the local Ethics Committee. All UC patients had total colitis with their disease lasting more than 7 years; they were therefore considered to be at high risk of developing cancer and/or dysplasia. Of these 53 patients, 10 had UC-associated neoplastic lesions, including 8 adenocarcinomas and 2 dysplasias (UC-Ca group). Forty-three UC patients had no neoplastic lesions (UC-NonCa group). In all UC cases, specimens were obtained from the nonneoplastic rectal mucosa for RNA extraction (Figure 1). Samples were collected either from surgically resected specimens or during colonoscopy. Samples were snap-frozen in liquid nitrogen and stored at −80°C until use. Parallel tumor specimens were formalin fixed and paraffin embedded for histologic examination. Samples were only used for RNA extraction when microscopic examination had verified that no neoplastic cells were present.

RNA Isolation and RT-PCR Using TaqMan Low Density Arrays

Total RNA was isolated from each of the frozen samples using RNeasy Mini Kit (QIAGEN) and gene expression levels were determined using TaqMan real-time PCR (Applied Biosystems) as described previously.[19] RNA quantity and integrity were checked by spectrophotometry and Bioanalyzer microfluidic analysis (Agilent Technology). First-strand cDNA was synthesized from total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) in 50 uL reaction volumes, using these cDNA samples for low density array (LDA) analysis according to the manufacturer's instructions. Each cDNA sample (100 ng per 2 uL) was added to 48 uL RNase-free water and 50 uL of 2× TaqMan Universal PCR Master Mix (Applied Biosystems). Final primer and probe concentration was 900 uM and 250 uM, respectively. The mixture was then transferred into a loading port on a LDA card. The card was centrifuged twice, sealed, and placed in an Applied Biosystems Prism 7900HT Sequence Detection System for PCR amplification using the following thermal cycler conditions: 2 minutes at 50°C and 10 minutes at 94.5°C for 40 cycles (30 seconds at 97°C and 1 minute at 59.7°C). Forty genes identified in our previous microarray analysis as predictive of patients at risk for developing UC-Ca were chosen and the corresponding validated primer/probe sets were incorporated into the LDA. The LDAs included *ACTB, B2M, GAPDH, GUSB, HMBS, RPLP0, YWHAZ,* and 18S as reference genes, based on their

Table 2 Classification of the Additional 149 Genes According to the KEGG Pathway Database

Pathways	No. of Genes within Each Pathway
Cytokine-Cytokine Receptors	29
Jak-STAT Signaling Pathway	22
MAPK Signaling Pathway	10
Pyrimidine Metabolism	10
Toll-like Receptor Signaling Pathway	9
Apoptosis	7
Cell Cycle	6
TGF-β Signaling Pathway	6
Purine Metabolism	5
Regulation of Actin Cytoskeleton	5
Amyotrophic Lateral Sclerosis (ALS)	4
Neurodegenerative Disorders	4
Wnt Signaling Pathway	3
Calcium Signaling Pathway	2
One Carbon Pool By Folate	2
Ascorbate and Aldehyde Metabolism	1
β-alanine Metabolism	1
Circadian Rhythm	1
Fluorine Degradation	1
Focal Adhesion	1
Folate Biosynthesis	1
γ-hexachlorocyclohexane Degradation	1
Glutathione Metabolism	1
Huntington's Disease	1
Limonene and Pinene Degradation	1
Neuroactive Ligand-Receptor Interaction	1
Nitrogen Metabolism	1
Notch Signaling Pathway	1
Pantothenate and CoA Biosynthesis	1
Parkinson's Disease	1
Porphyrin and Chlorophyll Metabolism	1
Prion Disease	1
Prostaglandin and Leukotriene Metabolism	1
Stilbene, Coumarine, and Lignin Biosynthesis	1
Tight Junction	1

Abbreviations: CoA = ; Jak-STAT = ; KEGG = Kyoto Encyclopedia of Genes and Genomes (*www.genome.jp/kegg/pathway.html*); MAPK = mitogen-activated protein kinase; Wnt = wingless/int-1.

proven role as housekeeping genes.[20,21] In addition to the 40 genes and transcripts previously identified, 149 new genes that had been reported to be important in carcinogenesis were also included in the LDA analysis (Figure 2). Table 2 shows a classification of the newly selected genes according to the pathways in which they function.

Data Processing and Statistical Analysis

To determine the stability of the selected reference genes, the geNORM algorithm (MS-Excel add-on-macro program) was used as described previously by Vandesompele et al.[22] This program calculates the gene stability measure M by determining the average pairwise variation between a particular reference gene and all other control genes. Using genes with M values below 1.5, a normalization factor was calculated based on the geometric mean of the expression levels of the selected genes. Relative gene expression was calculated by comparing ΔCT as previously described.[23] Normalized data were loaded into the GeneSpring software version 7.3 (Agilent Technologies). To identify discriminator genes, whose expression levels differed significantly between patients with and without UC-Ca, expression levels were compared using unpaired *t* tests and the Benjamini and Hochberg false discovery rate (FDR) control procedure.[24] Two-dimensional hierarchical clustering was then applied to the log-transformed data for the discriminator genes obtained by RT-PCR, and variations in multigene expression between patients with and without UC-Ca were compared by principal component analysis (PCA). The discriminator genes identified in these analyses were then used in supervised class predictions, using the support vector machine and a leave-1-out cross-validation as previously described,[13] to test their ability to distinguish UC-Ca and UC-NonCa patients.

Results

Comparison of Gene Expression Levels Between the UC-Ca and UC-NonCa Groups

Of the 176 genes and 13 transcripts (189 in total) used in the LDA analysis, 7 genes were housekeeping genes. After excluding these 7 genes, we finally selected 20 genes whose expression levels were significantly different in the UC-Ca and UC-NonCa groups ($P < .0052$; FDR P value $< .05$) (Table 3). As shown in Figure 3, all 20 genes showed a fold change greater than 1.5 between the 2 groups. Five genes showed higher and 15 genes lower expression levels in patients in the UC-Ca group compared with those in the UC-NonCa group. Of these 20 genes, the following 8 were included in the 40 predictor genes and transcripts analyzed in our previous microarray study[13]: *GBP4*, *SAMSN1*, *SLA*, *NOD27*, *SEPW1*, *EDIL3*, *NOL3*, and *LCP2*. Results of a hierarchical cluster analysis of the 20 genes are presented in Figure 4A. Visual inspection of the signal intensity map clearly showed the presence of a branch of the tree enriched for patients in the UC-Ca group (10 out of 18 patients), indicating that almost all patients were appropriately clustered into 2 distinct groups. We then used the 20 discriminator genes to generate a 3-dimensional plot of the data from a 20-dimensional plot (Figure 4B). PCA-based multidimensional scaling visualization separated samples in the UC-Ca and UC-NonCa groups into linearly separable gene expression data spaces.

Class Prediction for the Development of UC-Associated Neoplasm

Using the 20 discriminator genes as predictors, we performed supervised class prediction for the UC-Ca and the UC-NonCa groups using the support vector machine (SVM) and leave-1-out cross-validation.[25] We were able to predict the development of UC-associated neoplasm

Table 3 Twenty Discriminator Genes Distinguishing the UC-Ca and UC-NonCa Groups

Assay ID	Gene Symbol	Fold Change (UC/UC-Cancer)	*P* Value	Adjusted *P* Value (FDR *P*)	GenBank Accession Number	Description
Hs00180031_m1	IRF4	3.141	0.00145	0.0272	NM_002460	Interferon regulatory factor 4
Hs00223275_m1	SAMSN1	3.039	0.00109	0.0272	AF519621	SAM domain, SH3 domain, and nuclear localization signal 1
Hs00171065_m1	CXCL9	2.959	0.00194	0.0272	NM_002416	Chemokine (C-X-C motif) ligand 9
Hs00277129_m1	SLA	2.822	0.00115	0.0272	U44403	Src-like adaptor
Hs00188734_m1	PSCDBP	2.386	0.00166	0.0272	L06633	Pleckstrin homology, Sec7, and coiled-coil domain binding protein
Hs00252301_m1	SLAMF8	2.293	0.000459	0.0272	NM_020125	SLAM family member 8
Hs00164957_m1	ITGB2	2.256	0.00201	0.0272	L78790	Integrin, β2 (complement component 3 receptor 3 and 4 subunit)
Hs00258828_m1	URP2	2.223	0.00134	0.0272	BC004347	UNC–112-related protein 2
Hs00231709_m1	RUNX3	2.212	0.00306	0.0359	NM_004350	Runt-related transcription factor 3
Hs00175505_m1	LCP2	2.201	0.00412	0.0382	AI123251	Lymphocyte cytosolic protein 2 (SH2 domain containing leukocyte protein of 76 kDa)
Hs00168017_m1	CYP27B1	2.069	0.000698	0.0272	NM_000785	Cytochrome P450, family 27, subfamily B, Polypeptide 1
Hs00176719_m1	LYN	2.019	0.00516	0.0454	AI356412	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
Hs00168402_m1	IL2RB	1.847	0.00354	0.0373	NM_000878	Interleukin 2 receptor, β
Hs00364728_m1	GBP4	1.831	0.000429	0.0272	BG260886	Guanylate binding protein 4
Hs00260008_m1	NOD27	1.722	0.00177	0.0272	AA005023	Nucleotide-binding oligomerization domains 27
Hs00358724_g1	NOL3	0.566	0.00382	0.0373	BU785956	Nucleolar protein 3 (apoptosis repressor with CARD domain)
Hs00174781_m1	EDIL3	0.537	0.00269	0.0338	AA297258	EGF-like repeats and discoidin I-like domain 3
Hs00161621_m1	SEPW1	0.534	0.00194	0.0272	AW514401	Selenoprotein W, 1
Hs00609276_m1	GSN	0.499	0.00364	0.0373	NM_001127667	Gelsolin (amyloidosis, Finnish type)
Hs00193642_m1	PPP1R3C	0.251	0.00138	0.0272	N26005	Protein phosphatase 1, regulatory (inhibitor) subunit 3C

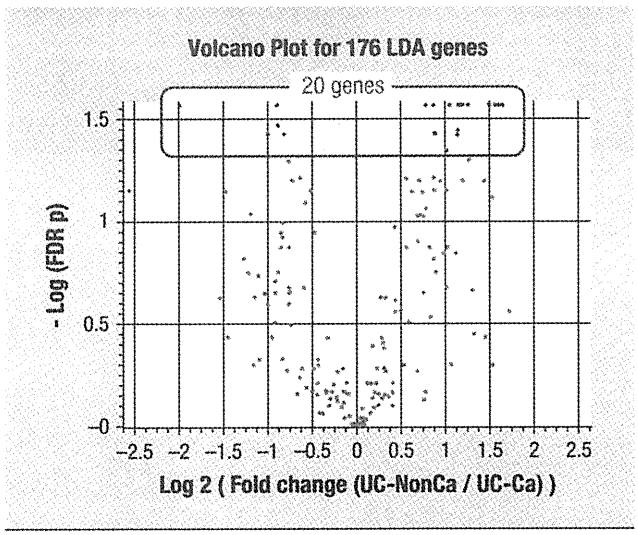
Abbreviations: Ca = cancer; EGF = endothelial growth factor; FDR = false discovery rate; ID = identifier; UC = ulcerative colitis.

with an accuracy of 83.0%. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were 100.0%, 79.1%, 52.6%, and 100.0%, respectively. Nine patients, all in the UC-NonCa group, were misclassified. To determine the number of genes that provided the best separation between patients with and without UC-Ca, we next ranked the 20 genes on the basis of the significance of their FDR *P* values and made predictions using 19 genes, 18 genes, and so on, starting from the bottom of the rank-ordered list. Figure 5 shows the prediction rates using different numbers of discriminator genes and shows that the greatest accuracy, 83.0%, was obtained using 16 to 20 of the top-ranked genes.

Discussion

Using quantitative RT-PCR analysis, we have been able to select 20 discriminator genes whose expression differed significantly between the UC-Ca and UC-NonCa groups. Two-way hierarchical clustering and PCA analyses using these 20 genes could distinguish patients in the UC-Ca and the UC-NonCa groups. In addition, based on the expression profiling of these 20 genes in nonneoplastic rectal mucosa by RT-PCR, we were able to build a predictive model which identified patients with UC-Ca or UC-NonCa with a high accuracy of 83.0%. This suggested that our predictive model may be useful for distinguishing patients with high and low risk for develop-

Figure 3 Volcano Plot Showing the Fold Change and *P* Values for 176 Genes. There were 20 genes with a fold change greater than 1.5



ing UC-Ca and this may lead to improvements in the efficacy of surveillance in UC patients. By stratifying patients according to their risk of UC-Ca, intensive surveillance could be targeted at high risk patients, and lower risk patients could receive less intensive surveillance. However, it is also important that this type of predictive model does not miss patients at high risk of developing UC-Ca. To achieve this goal a predictive model needs to show not only a high accuracy rate but also a high NPV. In our model the NPV was 100.0%, showing that it would be expected to identify all high risk patients correctly. These results suggest that our model may be useful for improving the efficacy of surveillance colonoscopy for UC patients in the clinic.

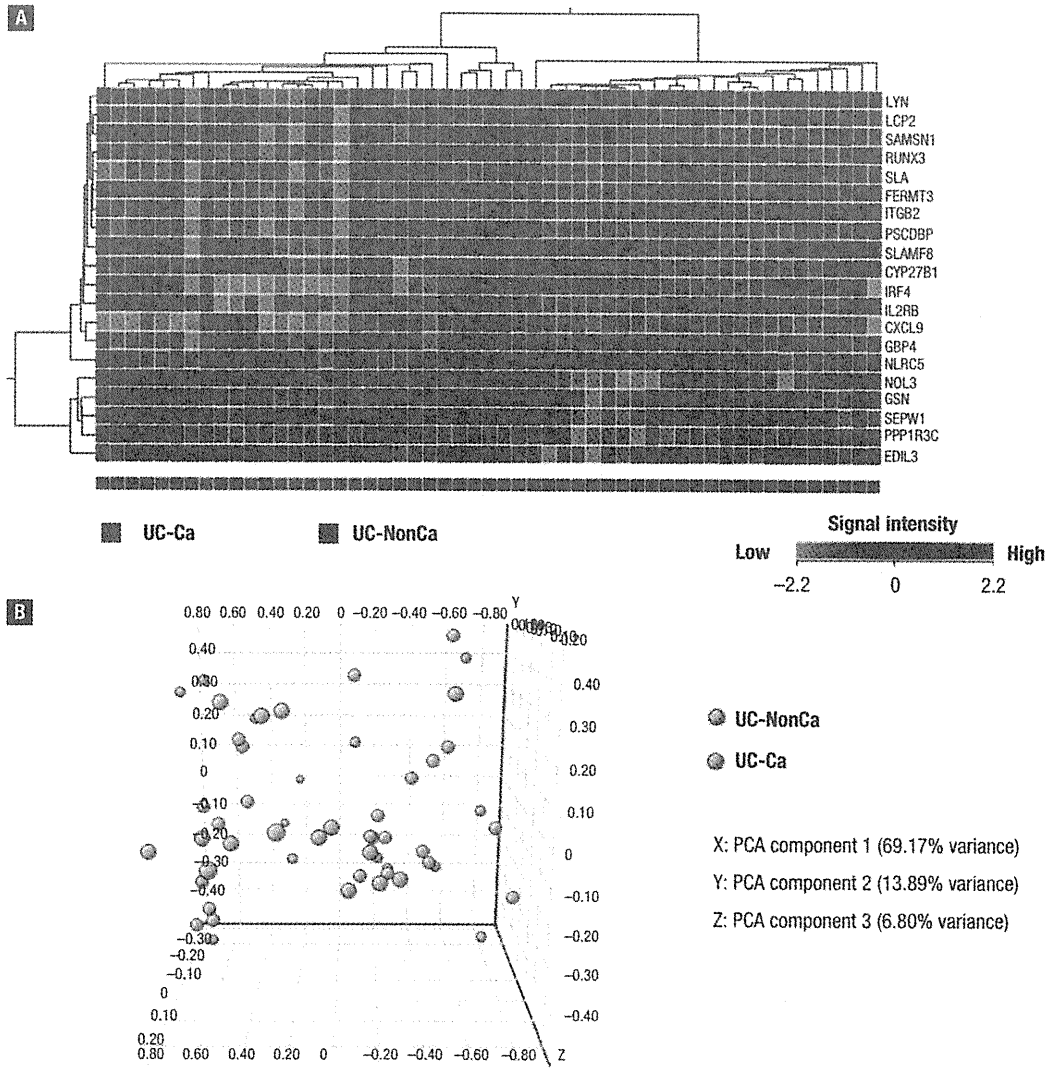
In our previous study, we demonstrated a predictive model for UC-Ca using a microarray. However, in a microarray analysis it is difficult to evaluate the expression of all genes because of the detection limit for each probe. Genes with low expression levels have to be excluded from the analysis by the data-cleaning process used. In fact, in our previous study, we had to exclude the expression data for 26,181 out of an original 54,675 probes in the final analysis. It is therefore possible that some important genes may have been excluded by this process. To overcome these problems associated with the use of microarrays, we included an additional 149 genes, which have been shown by others to be important in colon cancer carcinogenesis, in the present RT-PCR analysis. By analyzing a total of 189 genes and transcripts, we were finally able to select 20 discriminator genes whose expression differed significantly between the UC-Ca and UC-NonCa groups and could therefore be used in a predictive model.

Of these 20 discriminator genes, 8 were common to the genes identified in our previous microarray study. These genes included *SAMSN1*, *SEPW1*, *EDIL3*, and *NOL3*. *SAMSN1* maps to human chromosome 21q in a region that frequently shows loss of heterozygosity (LOH) in lung cancer, and a recent study reported that *SAMSN1* may act as a possible tumor suppressor gene for lung cancer.[26] *EDIL3* is a member of a family of extracellular matrix proteins with multiple endothelial growth factor (EGF)-like repeats.[27] The molecular function of the SEPW1 protein is unknown to date, but a role as an antioxidant has been proposed based on its ability to bind glutathione.[28] Other studies have demonstrated overexpression of EDIL3 in hepatocellular carcinoma and SEPW1 in breast cancer.[27,29] However, the roles of SAMSN1, EDIL3 and SEPW1 in colorectal carcinogenesis remain unknown. NOL3 an is inhibitor of apoptosis,[30] whose expression is markedly increased in the epithelium of primary human breast cancers compared with benign breast tissue and is also known to be high in colon cancers compared with adjacent benign colonic tissue.[30] Because the ability of cells to escape apoptosis is critical during carcinogenesis, NOL3 may play an important role in UC-associated carcinogenesis.

This study also selected 12 new genes that were not among the previous 40 predictor genes identified. These genes included *CYP27B1*, *RUNX3*, *CXCL9*, *ITGB2*, and *LYN*. *CYP27B1* plays an important role in calcium homeostasis by catalyzing the synthesis of the active form of vitamin D. Vitamin D prevents proliferation and induces apoptosis in colon cells, and an insufficiency of this vitamin is associated with an increased risk of colorectal cancer.[31] Ogunkolade et al. showed that concentrations of CYP27B1 mRNA were similar in colon cancer samples and in nonneoplastic colon samples from individuals without cancer, but were significantly lower in the nonneoplatic mucosa of patients with cancer.[31] They suggested that the downregulation of CYP27B1 could contribute to cancer risk. Our study has also shown that CYP27B1 expression in nonneoplastic colon tissue was significantly lower in patients with UC-Ca than in cancer-free patients. Together with the results from Ogunkolade et al., our results suggest that the downregulation of CYP27B1 may indeed indicate an increased cancer risk in UC-Ca patients. *RUNX3* is involved in the transforming growth factor β (TGF-β)-induced tumor suppressor pathway[32,33] and is known to act as a tumor suppressor gene in gastric cancer.[34] Previous studies reported that changes in *RUNX3* gene expression were also found in a number of cancers including breast, lung, hepatocellular, prostate, bile duct, pancreatic, and colon cancers.[32-34] However, to date, genetic changes in *RUNX3* have not been reported during UC-associated carcinogenesis and this study is the first to show the upregulation of RUNX3 expression in UC patients. CXCL9 is an antiangiogenic chemokine that has previously been shown to be overexpressed in colon cancer[35] and the expression of ITGB2 has been shown to correlate with clinical course of colorectal cancer after surgery.[36] LYN is a member of the Src family tyrosine kinases (SFK) and a negative regulator of apoptosis in various cell types. LYN plays an important role in cellular proliferation and metastasis in many human malignancies and the overexpression of LYN in colon cancer cell lines has been shown to induce chemoresistance.[37] Although these genes have been reported to be closely related to carcinogenesis in various cancers, a relationship to UC-Ca has not been reported previously. This study suggests that these genes may have important roles in UC-associated carcinogenesis.

One possible limitation of this study is the relatively small number of patients used, so that we could not validate the accuracy of our predictive model. To further confirm the accuracy of the model, we

Figure 4 Two-way Hierarchical Clustering and Principal Component Analysis. (A) Two-way hierarchical cluster analysis was used to order samples (columns) and array targets (rows). Red indicates overexpression, green indicates underexpression. Patients in the ulcerative colitis (UC)-associated cancer (UC-Ca) group and non-cancer (UC-NonCa) groups were clustered into 2 distinct groups. All patients were classified correctly on the basis of gene expression into either the UC-Ca or UC-NonCa group, except for 3 cases in the UC-NonCa group. At the bottom of the plot, red indicates the UC-Ca group and blue indicates the UC-NonCa group. (B) Principal component analysis. Discriminator genes were used to generate a 3-dimensional (from a 20-dimensional) plot of the data. principal component analysis (PCA)-based multidimensional scaling visualization separated samples in the UC-Ca (Red) and UC-NonCa (Green) groups into linearly separable gene expression data spaces

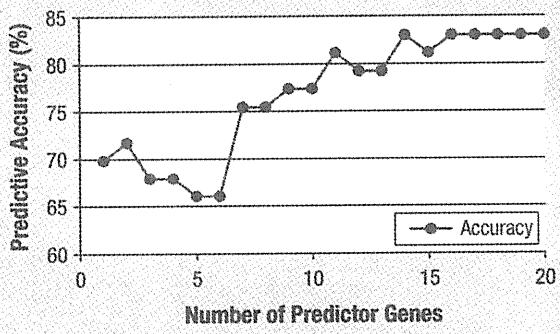


will need to validate the predictive value of the present model by use of an independent test set of patients to show clear robustness in a prospective trial. A second important point concerns the number of genes we have used for this predictive model. We derived this model using 20 discriminator genes, but in a clinical setting, using a smaller number of genes would be more practical. We therefore investigated using a smaller number of predictor genes and found that the same accuracy was obtained when we used 16, 17, 18, 19, or 20 of the top-ranked genes. This clearly suggested that we could reduce the number of predictive genes to 16. However, using any fewer predictor genes resulted in reduced accuracy for the predictions made by the model.

Conclusion

Based on the expression levels of 20 genes in the nonneoplastic rectal mucosa of UC patients, we have been able to predict the development of UC-Ca in patients with high accuracy. To our knowledge, this is the first study to show that RT-PCR analysis can be

Figure 5 The Accuracy of Predictions Using Different Numbers of Predictor Genes. The greatest accuracy (83.0%) was obtained using 20, 19, 18, 17, or 16 top-ranked genes



useful for accurately predicting the development of UC-associated neoplasms. This model may be useful for improving the efficacy of surveillance in UC. However, as the number of patients in this study was limited, the results need to be validated in a prospective study with a larger number of patients. Furthermore, the role of discriminator genes in UC-associated carcinogenesis needs to be clarified and this may provide useful directions for future research into the molecular mechanisms of UC-associated cancer.

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Disclosures

All authors have no conflicts of interest.

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ORIGINAL ARTICLE

The use of infliximab in the prevention of postsurgical recurrence in polysurgery Crohn's disease patients: a pilot open-labeled prospective study

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Abstract
Purpose Crohn's disease (CD) commonly recurs after surgery, and a number of patients need repeated surgery, especially smokers and those with repeated surgeries or penetrating disease. Whether infliximab prevents postsurgical recurrence in high-risk CD remains unknown. In the present pilot open-labeled study, we investigated the safety and efficacy of scheduled infliximab, which was started early after surgery, in maintaining remission of CD patients who have undergone multiple surgeries due to penetrating disease.
Methods Eleven patients (nine male, two female; age range, 26–48 years) who had undergone repeated surgeries (median, 4; range, 2–5) for penetrating disease were enrolled. Two to 4 weeks after surgery, the patients were started on intravenous infliximab (5 mg/kg) at an 8-week interval. The primary end points were the proportion of patients in clinical remission at the end of the study, the rate of endoscopic/radiologic remission at 24 months, and the rate of adverse effects.
Results One patient dropped out due to non-compliance, and ten patients were eligible for analysis. Clinical remission was maintained in six of ten patients (60.0%) at the end of the study. At 24 months, four out of ten patients were in endoscopic or radiological remission (40.0%). Two patients experienced adverse effects (18.2%), one of whom elected to withdraw from the study.
Conclusion The findings of no major safety concern and possible clinical benefit in our study suggest that further investigation of infliximab as a treatment for prevention of postsurgical recurrence in high-risk CD is warranted.

Keywords Crohn's disease · Infliximab · Maintenance · Postsurgical recurrence

Abbreviations

CD	Crohn's disease
IBD	Inflammatory bowel disease
5-ASA	5-Aminosalicylic acid
CDAI	Crohn's disease activity index

Introduction

Crohn's disease (CD) is a chronic inflammatory bowel disease (IBD) characterized by periods of remission interrupted by episodes of clinical relapse due to recurrent intestinal inflammation [1]. The pathogenesis of CD reflects dysregulated interaction among environmental factors, intestinal flora, and

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genetic susceptibility factors within the immune system, which triggers inflammatory activities in the intestinal mucosa [2–4]. Lesions of CD are characterized by trans-mural inflammation of potentially any section of the digestive tract, leading to various intestinal and extra-intestinal manifestations that often need surgery [3, 5].

The natural behavior of Crohn's disease is characterized by recurring active disease interrupted with periods of inactive disease and remission [5]. Approximately 75% of patients with CD require surgery during their lifetime. Surgery is not curative, since the disease almost invariably recurs due to the pan-enteric nature of CD. More than 80% of patients with CD develop endoscopic recurrence 1 year after surgery if they are not given any prophylactic treatment [6]. Clinical relapse occurs in approximately 50% of patients 5 years after surgery. The rates of recurrence requiring re-surgery have been reported to be 25% to 35% at 5 years and 40% to 70% at 15 years [7]. The risk for recurrence is higher for ileocolonic disease (50% at 5 years) than for ileal disease and higher for perforating disease than nonperforating disease [8, 9]. Furthermore, penetrating disease, multiple surgeries, and smoking have been reported as risk factors of disease recurrence. There is concern that reoperative surgery for Crohn's disease leads to a higher rate of major postoperative complications and ultimately the development of short bowel syndrome.

Conventional medications including 5-aminosalicylic acid (5-ASA), prednisolone (PSL), and immunomodulators like azathioprine/mercaptopurine have been used for many years in the treatment of active CD [10]. 5-ASA, metronidazole, and immunomodulators are also used for the maintenance of remission [11, 12]. However, a certain number of patients will experience relapse that requires surgery. Therefore, there is a need for effective and well-tolerated therapies for prevention of relapse in CD. Recently, anti-tumor necrosis factor agents, including infliximab, have been shown to be effective in inducing and maintaining remission in CD [13]. Several studies have shown that infliximab is effective in the prevention of postsurgical relapse in CD. Reguiero et al. evaluated whether infliximab reduces postoperative recurrence in CD in a placebo-controlled study [14]. They reported that infliximab therapy (started within 4 weeks of surgery and continued for 1 year) was effective in preventing endoscopic and histological recurrence at 1 year after surgery. Yamamoto et al. showed that infliximab had suppressive effects on clinical and endoscopic recurrence in patients with early endoscopic lesions after 6 months of surgical resection [15]. Both of the studies showed favorable outcome towards infliximab in the prevention of relapse after surgery. However, in those studies, only a small number of patients had undergone multiple surgeries before infliximab. Emerging data suggest that patients with multiple surgeries or penetrating disease and smokers are at high risk for postoperative CD recurrence [8, 9]. Whether infliximab can prevent recurrence in patients who are at high risk for re-surgery, such as those with a history of multiple surgeries and penetrating disease, remains elusive.

The present prospective pilot study was performed to evaluate the tolerability and efficacy of infliximab to prevent postsurgical recurrence in CD patients with a penetrating disease phenotype who have undergone multiple surgeries.

Methods

This pilot study was an open-labeled, prospective study aimed at evaluating the safety and efficacy of infliximab to prevent postsurgical recurrence in CD patients who have undergone multiple surgeries. The study period was from April 2003 to June 2008. The protocol and patients' informed consent forms were reviewed and approved by the hospital's Institutional Review Board. The study was conducted in accordance with the Declaration of Helsinki, in compliance with the consolidated Good Clinical Practice guideline and the applicable regulatory requirements.

Patient population

Male or nonpregnant female CD patients older than 12 years at Keio University Hospital, Kitasato Institute Hospital, and Kyousai Tachikawa Hospital who had undergone multiple surgeries (≧2) were eligible. The latest surgery had to be radical (i.e., it completely removed the macroscopically involved intestine). Patients were screened and, if they met the criteria below, were recruited to the study.

Preoperative assessments with colonoscopy and barium studies were required to rule out active disease outside the operated location. Patients without sufficient preoperative evaluation were excluded. Patients with leukopenia (leukocyte count <3,000/μL), serious heart or kidney disease, coagulation/liver disorder, and infection were excluded. Those who developed surgical or postsurgical complications such as ileus, wound infection, or anastomosis leakage were not recruited. Chest X-ray and Mantoux tuberculin skin test were performed to exclude tuberculosis infection. Oral maintenance treatment was permitted if given at a stable dose for at least 8 weeks before the surgery for 5-ASA and immunomodulators (mercaptopurine), and was continued at the same dose postoperatively. Patients already receiving infliximab before surgery were excluded. Doses of PSL were allowed to be tapered by the clinician's decision after the surgery. Concomitant medications, used for other diseases than CD, which did not violate the protocol inclusion, were allowed.

Treatment and follow-up

Infliximab (5 mg/kg) was started 2–4 weeks after surgery and continued every 8 weeks. Premedication of hydrocortisone (50 mg iv) was given to every patient before infusion. The duration of infusion of infliximab was 2 h in every case. With respect to the CD disease status, at each visit (every 8 weeks), diaries were reviewed, and clinical and laboratory assessments were performed to assess Crohn's disease activity index (CDAI).

Outcomes

Primary end point The primary efficacy parameter was the rate of patients maintaining clinical remission at the end of the study period (June 2008). Patients were defined as having a clinical relapse when they experienced a ≧50 increase in CDAI compared to baseline. This criterion was applied, since the majority of patients were having frequent bowel movements, despite curative surgery, which may have contributed to high CDAI at baseline. When patients withdrew due to adverse effects, they were defined as treatment failure as well.

Secondary end points The secondary efficacy parameter was the rate of endoscopic or radiological remission at 24 months and the cumulative risk of surgery before and after the introduction of maintenance infliximab therapy. The endoscopic score developed by Rutgeerts was used to assess endoscopic remission [6]. Briefly, the scores were as follows: i0, no lesions; i1, five or fewer aphthous lesions; i2, more than five aphthous lesions with normal mucosa between the lesions or skip areas of larger lesions or lesions confined to the ileocolonic anastomosis; i3, diffuse aphthous ileitis with diffusely inflamed mucosa; and i4, diffuse inflammation with large ulcers, nodules, and/or narrowing. Endoscopic remission was defined by a score of i0 or i1. For those patients who could not be assessed by ileocolonoscopy, a double-contrast barium small-bowel follow-through study was performed. The results were assessed by the performing radiologist, and patients were defined as remission, when there was no sign of active lesions due to Crohn's disease. When there were aphthous, discrete, or longitudinal ulcers, strictures, or any other lesion related to Crohn's disease, it was regarded as recurrence. Ileocolonoscopy and double-contrast barium follow through were performed and scored by a blinded physician. The incidence and severity of adverse side effects were also analyzed during the study period.

Statistical analysis

When appropriate, data are presented as mean±SE values. The survival curve was assessed by the Kaplan–Meier method, and the rate of surgery pre- and post-infliximab was assessed by the Mann–Whitney test. For statistical analysis, data were processed by using SAS software (SAS Institute, Cary, NC, USA), and graphs were created with GraphPad Prism (GraphPad Software, La Jolla, CA, USA). $P<0.05$ was considered statistically significant.

Results

Participant flow and follow-up

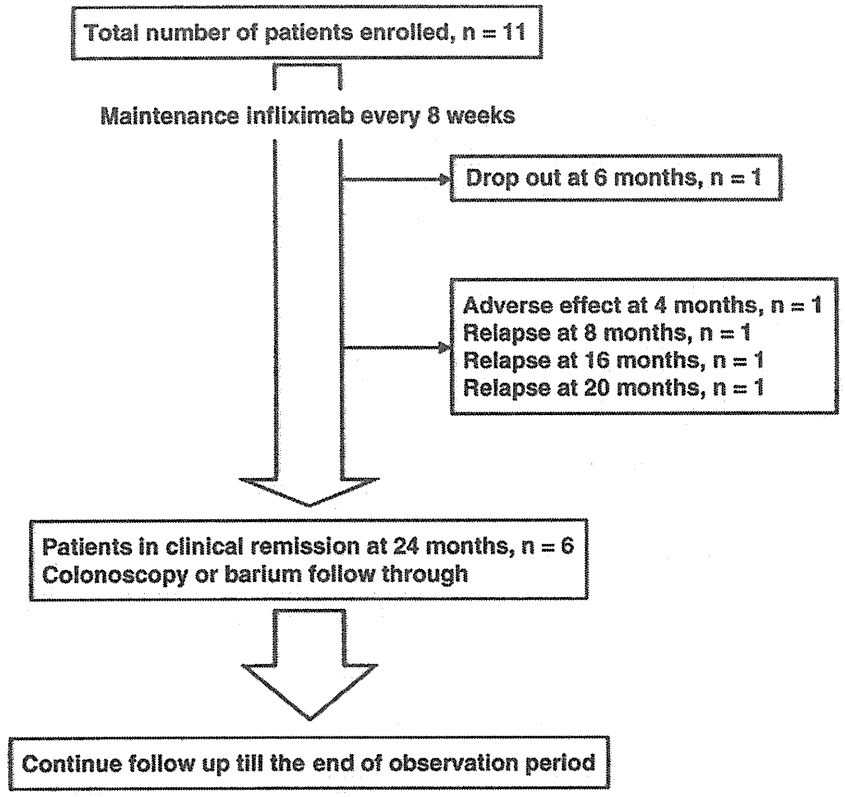
A total of 11 patients who had undergone two or more surgeries were enrolled in the study. The median number of surgery per patient was 4 (range, 2–5; mean, 3.36). Of the 11patients, none had operative or postoperative complications, and none was lost to follow-up. Nine patients (81.8%) were taking immunomodulators (mercaptopurine) preoperatively. The dose of mercaptopurine was 0.5 mg/kg which is the suggested dose in Japanese IBD patients defined in a previous study [16]. The two patients who were not taking mercaptopurine preoperatively had a history of intolerance in the past (nausea and liver dysfunction, respectively). Three patients were taking PSL preoperatively. The reasons for the most recent surgery were active disease with stricture and abscess in four, entero-cutaneous fistula in one, entero-cutaneous fistula with abscess in three, and entero-enteral fistula in three. Full patients' demography is presented in Table 1. Detailed characteristics of each patient are depicted in supplementary Fig. 1.

The flow chart of the study is shown in Fig. 1. One patient dropped out due to non-compliance after the third infliximab infusion (6 months) and withdrew from the study. Two patients developed infusion reactions (hypotension, dyspnea, and fever) at their second and third infusions, respectively. The former patient elected to withdraw from the study at 4 months. Treatment failure was defined as either disease relapse or withdrawal because of adverse effects.

Table 1 Baseline characteristics

Characteristics	
Sex (male/female)	9:2
Age (years, range)	34.1 (26–48)
Duration of CD (months, range)	134.4 (48–204)
Disease location (ileum/ileocolonic)	4:7
Anal lesions (*n*)	9
Number of previous surgeries (median, range)	4 (2–5)
Use of 5-ASA (*n*)	11
Use of steroids (*n*)	2
Use of immunomodulators (mercaptopurine) (*n*)	9
Smokers (*n*)	3

Fig. 1 Flowchart of the study



Primary efficacy evaluation

As stated above, one patient dropped out due to non-compliance at 6 months and was removed from analysis (Fig. 2). As per protocol, six out of ten patients (60.0%) maintained clinical remission at the end of the study. The median follow-up period was 33 months (range, 4–65). One patient relapsed at 8 months, due to an entero-enteral fistula accompanied with bowel obstruction, and required re-surgery. This patient also developed an infusion reaction. One patient relapsed at 16 months due to a bleeding ulcer at the ileocolonic anastomosis. Another patient developed a gastric ulcer at 20 months which upon immunohistochemistry showed granulomas and was diagnosed that it was caused by Crohn's disease. Prior evaluation did not show any upper gastrointestinal lesion in this patient, and it was presumed that this lesion developed during infliximab treatment. Both of the patients were given three induction infusions (0, 2, 6 weeks) of infliximab followed by maintenance infusions every 6 weeks to re-induce and maintain remission. One patient who developed an infusion reaction at her second infusion (4 months) refused to continue the study.

Secondary efficacy evaluation

Six patients who had maintained clinical remission either underwent ileocolonoscopy or double-contrast barium follow through by a blinded physician at 24 months. Of the four patients who underwent ileocolonoscopy, one patient had multiple small ulcers on both sides of the ileocolonic anastomosis (i2), and another patient developed a fistula at the ileocolonic anastomosis. The two patients who underwent double-contrast barium follow through had no active lesions. As per protocol, four out of ten patients (40.0%) were in endoscopic or radiological remission at 24 months.

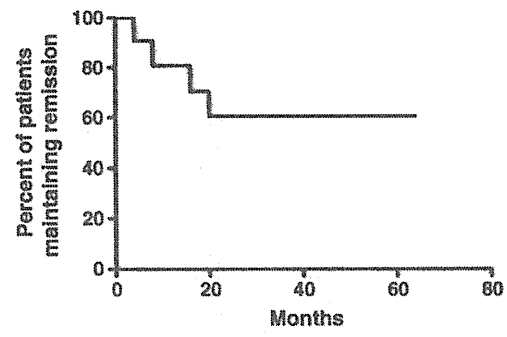


Fig. 2 Changes in the proportion of patients in clinical remission by maintenance infliximab shown with the Kaplan–Meier method. One patient dropped out due to non-compliance at 6 months and was removed from analysis. During the follow-up period (mean, 26.8 months; range, 4–65), six of ten patients (60.0%) maintained remission. One patient relapsed at 8 months, due to an entero-enteral fistula accompanied with bowel obstruction and required re-surgery. One patient relapsed at 16 months due to a bleeding ulcer at the ileorectal anastomosis. Another patient relapsed at 20 months due to a bleeding giant gastric ulcer caused by Crohn's disease. One patient withdrew from the study at 4 months due to infusion reaction

The cumulative risk of surgery before the current study was 0.43/patient-year (95% confidence interval (CI) 0.26 to 0.59) in the present patient population (Fig. 3). The risk decreased to 0.21/patient-year after the initiation of maintenance infliximab treatment (95% CI 0.30 to 0.73, P=0.015). This suggests that infliximab, if given early after surgery, may prevent re-surgery in patients who are at high risk of relapse.

Regarding safety and tolerability, two patients experienced infusion reactions (18.2%), one of whom refused to continue the study, and the other was successfully treated with acetaminophen and chlorphenylamine. One patient (9.09%) dropped out due to non-compliance after the third infliximab infusion (6 months), though he did not have any side effects. No serious infection or malignancy occurred during the study period. However, one patient (9.09%) experienced herpes zoster infection after the sixth infliximab infusion, which was successfully treated with oral valaciclovir.

Discussion

The present pilot study was performed to seek the efficacy and tolerability of scheduled infliximab in maintenance of remission of CD patients with a penetrating disease phenotype who have undergone multiple surgeries. We have shown that maintenance treatment with infliximab prevented clinical and endoscopic/radiological recurrence in 60% and 40% of patients who are at risk for recurrence, respectively.

Recently, several studies have shown that infliximab is effective against postoperative recurrence of CD. Reguiero et al. reported that infliximab prevented endoscopic recurrence at 1 year in more than 90% of patients [14]. Sorrentino et al. reported that patients treated with infliximab and low-dose methotrexate showed no endoscopic or clinical recurrence after 2 years [17]. No patient required re-surgery in their studies. In the present study, one patient required re-surgery due to recurrence of CD, and 60% of patients had maintained a clinical remission, which are contradictory to the previous reports. The patient population in our study

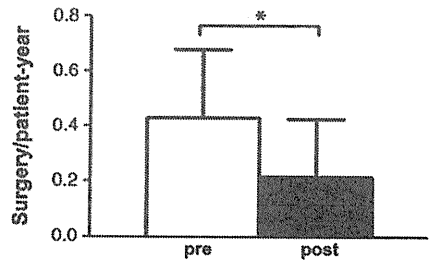


Fig. 3 Changes in the cumulative numbers of surgeries before and after the introduction of maintenance infliximab therapy. The cumulative risk of surgery before the current study was 0.41/patient-year in the present patient population. The risk decreased to 0.21/patient-year after the induction of maintenance infliximab treatment (P=0.015)

may have carried a higher risk of recurrence; however, the cumulative risk of surgery in our series of patients still decreased from 0.43/patient-year before the current study to 0.21/patient-year after the introduction of maintenance infliximab. This suggests that infliximab, if given early after surgery, may prevent re-surgery in patients who are at high risk of recurrence.

In the present pilot study, we only included patients with penetrating disease phenotype who had a history of multiple surgeries. This population carries higher risk of disease recurrence and needs proper prophylaxis to avoid further surgery to prevent future complications, such as short bowel and malnutrition. Smoking and female gender are also risk factors for postsurgical recurrence, and, in fact, three of the patients who smoked did continue to do so after the surgery. One of the smokers and one of the females experienced recurrence, respectively, which may have affected the results of our study. Infliximab was administered 2 to 4 weeks after surgery and subsequently continued every 8 weeks. We chose this regimen, because it has been reported that endoscopic recurrence may occur early after surgery, and the main purpose of this study was to assess the efficacy of infliximab in maintaining surgically achieved remission, but not inducing remission. Thus, we started infliximab at an 8-week interval and not with the 0-, 2-, and 6-week induction schedule as in Reguiero's study [14]. Administering infliximab with an induction schedule may improve outcome and should be considered in future studies. We did not check serum infliximab level or antibody against it in the current study; however, this issue should be taken into account in future studies, since they may guide subsequent treatment decisions.

The recurrence rates were relatively higher in our patient population compared to previous studies; however, the history and characteristics of our patient population suggest that without proper prophylaxis, they were highly likely to experience recurrence and re-surgery. One explanation for the discrepancy in the recurrence rate is that we only included patients who have undergone multiple surgeries due to perforating disease, who may have had higher risk of recurrence. Furthermore, the follow-up period was longer in our study, which may have also accounted for the higher recurrence rate. Majority of the patients (9 out of 11) continued the preoperative dose of mercaptopurine in combination with infliximab to achieve as much clinical benefit as possible. Though the dose of mercaptopurine used in our patient population was low, we have previously confirmed its effectiveness in Japanese patients with IBD.

We are also aware that our pilot study only included 11 patients, and it is difficult to draw a firm conclusion from the results of our study. The number of participants was limited, since our inclusion criteria provided that they required multiple surgeries, penetrating disease phenotype, and should

not have had any postsurgical complications such as bowel obstruction, prolonged fever, wound infection, etc. Since our study was a pilot study, it also lacks a control group, such as placebo-treated patients. To confirm whether infliximab can prevent postsurgical recurrence in the current patient population, a larger comparative study with a placebo-treated control group is necessary.

The results of our study showed that postoperative scheduled infliximab maintained clinical remission and endoscopic/radiologic remission in 60% and 40% of the patients, respectively. Paradoxically, however, the results of our study also show that around half of the patients experience either clinical or endoscopic/radiologic recurrence after surgery despite prophylaxis with infliximab. This reminds us about the limitations of infliximab in preventing postsurgical recurrence in high-risk patients and the further need to seek a more efficacious prophylaxis.

In conclusion, we have conducted a pilot study to assess the tolerability and efficacy of maintenance infliximab treatment in CD patients, with a penetrating disease phenotype who had undergone multiple surgeries and who appeared to be at high risk for postsurgical recurrence. Though our study population was small, it showed that infliximab was well tolerated and prevented recurrence in more than half of the patients. This indicates that infliximab, if given early, may be capable of changing the natural history of CD patients who are at high risk of recurrence. A larger-sized, randomized, controlled study to firmly establish the safety and efficacy of infliximab in the prevention of recurrence of high-risk CD is warranted.

Conflict of interest TS, HM, SO, HT, HO, and YI have no conflict of interest. AS received research support from Mitsubishi-Tanabe Pharmaceutical Co. TH serves as a consultant for Mitsubishi-Tanabe Pharmaceutical Co.

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ORIGINAL ARTICLE

Double-Contrast Barium Enteroclysis as a Patency Tool for Nonsteroidal Anti-Inflammatory Drug-Induced Enteropathy

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Abstract
Background Evaluating small bowel patency is recommended for capsule endoscopy in patients suspected of nonsteroidal anti-inflammatory drug-induced (NSAID) enteropathy.
Aims The aim of this investigation was to examine whether radiography is a candidate of patency tool in NSAID enteropathy.
Methods We reviewed double-contrast barium enteroclysis in 21 patients with NSAID enteropathy diagnosed either by capsule endoscopy or balloon-assisted endoscopy. The endoscopic findings were classified into circular ulcers, linear ulcers and small mucosal defects. The radiographic signs of the corresponding endoscopic findings were retrieved and the depiction rate was calculated.
Results Of the 21 patients, endoscopy detected circular ulcers, linear ulcers, and small ulcers in 12, 3 and 12 patients, respectively. Small bowel radiography depicted circular narrowing as pseudo-folds in 10 patients (83%) and linear ulcers as eccentric rigidity in 2 patients (67%). However, radiography was able to depict small mucosal defects in only 3 patients (17%). Two of 5 patients with pseudo-folds experienced retention of the capsule.
Conclusion "Pseudo-folds" is a sign corresponding to circular ulcer in NSAID enteropathy, which may be predictive of capsule retention.

Keywords Nonsteroidal anti-inflammatory drug · Small bowel · Capsule endoscopy · Balloon-assisted enteroscopy · Radiography

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Introduction

It has become evident that patients taking nonsteroidal anti-inflammatory drugs (NSAID) are at high risk of small bowel mucosal lesions [1, 2]. In observational studies by means of video-capsule endoscopy (VCE) or balloon-assisted endoscopy (BAE), more than 50% of patients under long-term NSAID use had small bowel ulcers [3–6]. The endoscopic findings of NSAID enteropathy vary widely, from diminutive mucosal defects to sharply demarcated ulcers [3, 4, 7–9]. Amongst various types of mucosal lesions, severe and concentric strictures of the small bowel, referred to as diaphragms, are the most characteristic of NSAID enteropathy [10, 11].

In the early period after the introduction of CE, cases of NSAID enteropathy with the diaphragms were examined by the procedure, and as a consequence, those cases suffered from capsule retention [12, 13]. It has subsequently been reported that NSAID enteropathy is one of the major causes of the retention of the capsule. In a single center analysis, Li et al. [14] reported that 8 of 14 patients who experienced capsule retention had NSAID enteropathy. In an extensive review by Liao et al. [15], NSAID enteropathy has been shown to be the third most frequent cause of capsule retention, accounting for 18.4% of such cases.

In order to avoid capsule retention, a patency capsule system has been developed and become clinically available [16–18]. However, the patency system is time-consuming, requiring 5 days for final decisions at most [18]. Furthermore, it is a historical fact that diaphragms could be diagnosed by small bowel radiography in the 1990s [19–22]. We thus made a retrospective analysis of our patients with endoscopically diagnosed NSAID enteropathy to assess the role of double-contrast barium enteroclysis as a procedure for luminal patency in the disease.

Methods

Patients

We reviewed patients with small bowel ulcers detected by VCE or BAE at our institutions during the period 2003–2011, and identified 53 patients who fulfilled the following criteria for NSAID enteropathy. The criteria included (1) presence of small bowel mucosal lesions, (2) NSAID intake for at least a week just prior to enteroscopy, and (3) ulcer healing after discontinuance of the NSAID without any specific treatment. Among the patients, we recruited 21 who had been examined by double-contrast barium enteroclysis (DCBE) prior to enteroscopy for the present investigation. Written informed consent was obtained from each subject with regards to the purpose and the method of each examination. This retrospective study was undertaken according to the Helsinki Declaration.

There were 7 females and 14 males, and the ages at the time of enteroscopy ranged from 46 to 88 years (mean, 68 years). The indication for enteroscopy was overt obscure gastrointestinal bleeding (OGIB) in 13 patients, abdominal pain in 5, and occult OGIB in 3. Seventeen patients had been taking a single NSAID, while 4 patients were under two species of NSAID. The species of NSAID were loxioprofen (7 patients), diclofenac (6), low-dose aspirin (4), indomethacin (2), meloxicam (2), ibuprofen, naproxen, ampyroxicam and celecoxib (each in 1 patient). The indication for NSAID use was osteoarthropathy in 10 patients, rheumatoid arthritis in 6, other arthralgia in 2, and cardiovascular diseases in 3. Time duration from the start of NSAID until the diagnosis of the enteropathy ranged from 0.2 to 240 months with a mean of 52 months.

Enteroscopy

VCE was performed by either PillCam SB system (Given Imaging, Yoqneam, Israel) or EndoCapsule system (Olympus, Tokyo, Japan), according to the manufacturer's recommendation. After an overnight fast, patients were prepared by simethicone with tap water or 900 ml of magnesium citrate prior to the examination [23]. Patients were then instructed to ingest the capsule and the images for the subsequent 8 h were recorded. The VCE images were reviewed by one of the authors (M.E.), who was informed of the patients' characteristics including NSAID use. Capsule retention was regarded as a case of retained capsule for more than 3 days, which required subsequent endoscopic or surgical procedure for removal.

Oral and anal BAEs were performed with Double Balloon Enteroscopy System (Fujifilm, Tokyo, Japan) under fluoroscopy [24]. After an overnight fast, the patients were prepared by 2 l of electrolyte lavage solution in cases of anal BAE. The route for BAE was determined by the endoscopists on the basis of the patients' characteristics. The patients were prepared by continuous intravenous infusion, and examined by enteroscopy under a light sedation by intravenous midazolam. The scope was advanced as far as possible with reciprocal insertion of the scope and the overtube.

The VCE and BAE findings were classified into circular ulcers, linear ulcers, and small mucosal defects [7]. A severe, concentric stenosis with diaphragm was regarded as circular ulcers. Small mucosal defects included red spots and small ulcers.

Double-Contrast Barium Enteroclysis

Small bowel radiography was performed with a double-contrast technique as has previously described [25]. In brief, patients were prepared by an insertion of a naso-jejunal tube under fluoroscopy. The tube was fixed at the ligament of Treitz by a pneumodilatation of the balloon at the tip of the tube. Then, 200–300 ml of 70% v/w barium sulphate was slowly injected through the tube until the terminal ileum was filled with the contrast material. The small intestine was then inflated with 800–1,000 ml of air injected through the tube. When a sufficient inflation was achieved, 40 mg of scopolamine butyl bromide was injected intravenously to inhibit peristalsis and to obtain double-contrast images.

Assessment

We first compared clinical features between patients with and without each endoscopic finding. Radiographic images were then reviewed by two enteroscopists (T.M. and M.E.) with a reference to each enteroscopic finding. The depiction rate of each enteroscopic finding was calculated.

Statistical Analyses

When comparing two groups, Mann–Whitney test, chi-squared test or Fisher's exact probability test were used