Microarray analyses of peripheral whole blood cells from ulcerative colitis patients: Effects of leukocytapheresis

TEPPEI KOBAYASHI, KEIICHI MITSUYAMA, HIROSHI YAMASAKI, JUNYA MASUDA, HIDETOSHI TAKEDATSU, KOTARO KUWAKI, SHINICHIRO YOSHIOKA, KOJI NAGAYAMA and MICHIO SATA

Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, Kurume 830-0011, Japan

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Abstract. Complementary DNA microarray technology allows the simultaneous analysis of the expression of hundreds to thousands of genes. We applied this technique to clarify the molecular mechanisms underlying the therapeutic effects of leukocytapheresis (LCAP) therapy in patients with ulcerative colitis (UC). A 776-gene microarray analysis was performed using whole blood cells from six normal subjects and six patients with active UC who had undergone filtration LCAP. Widespread gene upregulation was observed in patients with UC, compared with normal subjects. After LCAP, genes with proinflammatory actions, such as CD97, CD74, human leukocyte antigen-DR β 1 and -DP light chain, were downregulated, while genes responsible for antimicrobial actions, such as neutrophil gelatinase-associated lipocalin, and acute phase reactions, such as haptoglobin alS and al-acid glycoprotein, were upregulated. In conclusion, we identified several genes expressed in the whole blood cells of UC patients as well as the transcriptional events following LCAP. Following LCAP, the gene profile shifted toward a pattern indicating disease improvement. These results suggest a basis for the molecular mechanisms leading to the therapeutic effects of LCAP and also indicate new therapeutic targets, providing important prognostic information.

Introduction

Ulcerative colitis (UC), a type of inflammatory bowel disease (IBD), is a complex clinical entity in which genetic, environmental and microbial factors interact to determine the susceptibility response of immune and non-immune cellular systems mediating inflammation (1-3). Although the precise mechanisms of pathogenesis remain unclear, studies using radiolabeled leukocytes (4) or immunohistochemical techniques (5) have revealed evidence of the recruitment of circulating leukocytes to the affected bowel. During active disease, newly migrated leukocytes from the circulation may be exposed to bacteria in the gut lumen, which may further activate these cells and result in severe mucosal damage. The removal of circulating leukocytes was therefore speculated to be an attractive approach for treating UC.

Trials of therapeutic leukofiltration from the peripheral circulation, otherwise known as leukocytapheresis (LCAP), have been performed using a veno-venous extracorporeal apheresis device coupled to a leukofiltration device (6,7). In several trials, LCAP appeared to attenuate inflammation in patients with UC (8-11) as well as patients with Crohn's disease (12), rheumatoid arthritis (13,14) and rapidly progressive glomerulonephritis (15), without provoking severe complications. Summarizing the results of previous clinical reports, Ortolano *et al* (8) concluded that 76% of 115 patients with IBD who were treated using LCAP entered remission, obviating the need for ongoing corticosteroid or cytoablative support. However, the mechanisms underlying these therapeutic effects have not been fully clarified.

As a breakthrough technology in the development of modern functional research in the field of human genomebased science, complementary DNA (cDNA) microarrays have become a powerful and sensitive technique that can be broadly applied to both basic and clinical research. This method makes it practical to quantitatively and simultaneously measure the expression levels of a large number of genes and has been successfully used to observe alterations and variations in gene expression in a variety of cells and tissues. In three previous studies, gene expression in IBD tissue, either from patients with UC or Crohn's disease, was compared with that in noninflamed or inflamed control tissue using cDNA microarrays (16-18). These experiments resulted in the identification of a large number of genes that are differentially expressed in the mucosa of patients with IBD, including genes that have been previously associated with IBD as well as candidate genes that have not been previously associated with IBD (16).

Correspondence to: Dr Keiichi Mitsuyama, Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, Asahi-machi 67, Kurume 830-0011, Japan E-mail: ibd@med.kurume-u.ac.jp

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In the present study, we used cDNA microarrays to investigate, for the first time, the global gene expression profiles of whole blood cells from patients with UC who had received LCAP. Our results suggest a basis for the molecular mechanisms leading to the therapeutic effects of LCAP and indicate new therapeutic targets, providing important prognostic information.

Materials and methods

Characteristics of subjects. The characteristics of the normal subjects and patients examined in this study are shown in Table I. Six patients with active UC were enrolled (3 men, 3 women; mean age, 27.5 years; mean disease duration, 6.2 years). Patients had either left-sided colitis (n=3) or pancolitis (n=3) and were classified into the moderate (n=3)or severe (n=3) attack categories, according to the Truelove and Witts criteria (19). All patients received standard medical therapy with aminosalicylates and/or corticosteroids. Patients with any of the following features were excluded from the study: an age <18 or >80 years, a serum hemoglobin <8 g/dl, a total leukocyte count $<4,000/\mu$ l, or coagulation abnormalities, bleeding diathesis, pregnancy or unsuitable peripheral venous access for apheresis. The apheresis procedure was well tolerated by all the patients. No severe complications occurred during the procedure. Informed consent was obtained from all the patients prior to undergoing LCAP. Blood samples from age- and gender-matched healthy volunteers were examined as normal controls.

LCAP procedure. LCAP was performed using a Cellsorba E column (Asahi Kasei Medical, Tokyo, Japan) installed in the extracorporeal circulation system (Plasauto LC; Asahi Kasei Medical) (6,7). For apheresis, venous access was secured via two large peripheral veins, and the blood was anticoagulated with nafamostat mesilate (Torii Pharmaceutical, Tokyo, Japan), a protease inhibitor that inhibits the activity of coagulation factors and platelet aggregation (6,7,9-15). Heparin was not used, since its use has been associated with respiratory distress and palpitations (20). With a flow rate of 30-50 ml/min for 60 min, a total of ~3 liters of blood was treated during each session. In principle, the LCAP procedure was carried out weekly for 5 weeks.

Preparation of RNA from blood. Samples (2.5 ml x2) of whole blood were drawn into PAXgene Blood RNA tubes (Qiagen, Hilden, Germany), and total RNA was extracted and purified according to the manufacturer's instructions. The quantity of RNA obtained from the extraction step was assessed using a NanoDrop ND-1000 instrument (NanoDrop Technologies, Wilmington, DE). The quality of the extracted RNA was determined using a Bioanalyser 2100 (Agilent Technologies, Palo Alto, CA, USA). A ribosomal RNA 28S/18S ratio above 1.3 was verified for all experiments.

Preparation of the cDNA microarray. We designed and prepared a low-density cDNA microarray for mRNA expression profiling in whole blood. Genes for this microarray were selected from the public database of SAGE results (http://133.11.248.12/; homepage of Department of Molecular Preventative Medicine, School of Medicine, University of Tokyo) prepared from activated blood cells, such as T cells, dendritic cells, monocytes, and macrophages (21-24). As described previously (24), a total of 776 genes were spotted onto SuperAmine (Telechem International, Sunnyvale, CA, USA) in quadruplicate, along with positive and negative control genes. For most of the genes, each cDNA was designed to be \sim 500 to 600 bp and to be within \sim 1 kb from the 3'-poly(A) tail. In addition, all cDNAs for the microarray probe were cloned into the pGEM vector (Promega, Madison, WI, USA). All clones for the capture probe were sequenced and validated by comparison with the GenBank sequence. In some cases, the 776 genes were divided into 20 groups based on their functional relatedness: lymphokine/cytokine/chemokine-related, cell surface antigen/immune response-related, kinase/kinase inhibitor-related, apoptosis/stress-related, matrix-related/ membrane-bound-related, metal-related, oncogene/suppressorrelated, cell cycle/transcription factor-related, DNA/ RNA-binding protein-related, energy/metabolism-related, drug metabolism-related, protease-related, serum protein/ anti-coagulation-related, proteasome-related, autoimmune system-related, general enzyme-related, receptor-related, ribosomal protein-related, miscellaneous, and control, according to the Atlas cDNA Expression Arrays (Clontech Laboratories, Palo Alto, CA, USA).

Reference RNA. Reference RNA was established from a mixture of whole blood (drawn into PAXgene tubes) samples obtained from healthy volunteers. The extracted total RNA, which was certified to be of sufficient quality using the Agilent RNA chip, was amplified using the MessageAmp aRNA kit (Ambion, Austin, TX, USA) to generate amplified RNA (aRNA). External non-human artificial RNA (*C. elegans* Y49G5B fragment) was spiked into the reference aRNA to distinguish it from the sample aRNA.

Preparation of sample RNA, labeling, hybridization and scanning. Total RNA (1 mg) from subjects was transcribed and amplified into aRNA using the MessageAmp aRNA kit, according to the manufacturer's instructions. Next, an external control RNA mixture [λ DNA (LD)], the baculovirus glycoprotein gene (GP), and the Renilla luciferase gene (RL); 9 mg each) were added to both the sample and reference aRNAs. The sample and reference aRNAs were then labeled with Cy5-dUTP and Cy3-dUTP (Perkin-Elmer, Boston, MA), respectively, using a SuperScript II kit (Invitrogen, Carlsbad, CA, USA) together with random hexamers (Takara, Kyoto, Japan). Competitive hybridization of the Cy3-labeled reference and the Cy5-labeled sample cDNAs on the microarray was carried out using a chamber system (Agilent Technologies), according to the method described by Khodursky et al (25). Slides were scanned five times at five different power ranges using a ScanArray 5000 (Perkin-Elmer). For further statistical analysis, the data was converted from tiff image data to signals using ImaGene software (Biodiscovery, Inc., El Segundo, CA, USA). The data files for the five scans were merged to establish a single representative data set for each gene (subject pending PCT/JP03/06677). The Cy5 (subject sample)/Cy3 (reference sample) ratio for each mRNA signal was calculated using global Lowess normalization (26).

Patient no.	Age (years)/ gender	Disease duration (years)	Disease extent	Disease activity ^a	Concomitant medications (mg/day)	
					Prednisolone	5-Aminosalicylic acid
1	18/F	0.3	Entire colon	Severe	40	2000
2	22/M	1	Left-side colon	Severe	10	1250
3	23/F	7	Left-side colon	Moderate	10	2250
4	25/M	5	Entire colon	Moderate	15	2250
5	31/F	9	Left-side colon	Severe	30	2250
6	46/M	15	Entire colon	Moderate	5	2250
^a Based on t	he criteria of Truelov	ve and Witts.				

Table I. Characteristics of the UC	patients providing sam	ples for the cDNA microarray	assay.
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Table II. Cell counts in the peripheral blood of the normal subjects and the patients with ulcerative colitis.

			Ulcerative colitis (n=6)		
		Normal subjects (n=6)	Before LCAP	After LCAP	
Leukocytes	(/µl)	5283±987	5900±1173	8550±4142	
Neutrophils	(/µl)	3093±1067	3780±1708	6970 ± 4392^{d}	
	(%)	57.3±12.0	61.8±17.9	$78.2 \pm 11.2^{a,d}$	
Eosinophils	(/µl)	123±54	172±182	124±112	
	(%)	2.4±1.2	3.5±4.1	2.1±2.0	
Basophils	(/µl)	59±47	36±40	41±65	
	(%)	1.2±1.1	0.7±0.7	0.4±0.5	
Lymphocytes	(/µl)	1760±601	1608±550	1285±553	
	(%)	34.3±11.8	28.7±11.8	$17.8 \pm 9.4^{a,d}$	
Monocytes	(/µl)	240±120	292±193	123±78	
	(%)	4.6±2.1	5.2±3.4	$1.5 \pm 1.1^{a,d}$	
Platelets	$(10^4/\mu l)$	19±4	30±11 ^a	16±5 ^e	
Erythrocytes	$(10^4/\mu l)$	479±21	378±52 ^b	330±41 ^{c,e}	
^a P<0.05, ^b P<0.0	05, °P<0.0001 vs. no	ormal subjects; dP<0.05, P<0.005 vs. UC befor	ore LCAP.		

Statistical analysis. The statistical analysis was performed using a t-test or paired t-test. Results were expressed as the mean and SD. P-values of <0.05 were considered to indicate a statistically significant result.

Results

Peripheral white blood cell count. Table II shows the peripheral white blood cell count in the six normal subjects and the six UC patients before and after LCAP. The absolute number and proportion of leukocyte subsets were similar between the UC patients and the normal subjects. Interestingly, the proportion of neutrophils increased and the proportion of lymphocytes and monocytes decreased in UC patients after LCAP.

Overall gene expression profiles. The cDNA microarray analyses of whole blood cells from normal subjects, UC

patients before LCAP, and the same UC patients after treatment repeatedly showed heterogeneous signals (Fig. 1). Two genes were uniquely upregulated by >2-fold and no genes were uniquely downregulated by >2-fold in normal subjects, whereas five genes were uniquely upregulated and two genes were uniquely downregulated in UC patients before LCAP, and 19 genes were uniquely upregulated and 18 genes were uniquely downregulated after LCAP.

Gene expression profiles: UC patients vs. normal subjects. Fig. 2A shows the gene expression profiles of whole blood cells from UC patients vs. those from normal subjects. The genes that were upregulated by >2-fold in the UC patients are summarized in Table III. These genes include galactoside-binding 3 (galectin 3), immunoglobulin heavy constant γ 3, human mRNA for calcium-binding protein in macrophages (MRP-14), γ -G globin (HBG2), and interleukin-1 receptor type II (IL-1R2). In



Figure 1. Global gene expression profiles of (A) normal subjects, (B) of UC patients before LCAP, and (C) of patients after LCAP. The vertical bars indicate an increase or decrease in gene expression in patients with UC, compared with gene expression in the control subjects, at a 2-fold level of the Cy5 (subject sample)/Cy3 (reference sample) ratio for each mRNA signal. 'Downregulated' designates genes that are downregulated, compared with the control profile at a 2-fold level, 'upregulated' indicates genes that are upregulated, compared with the control profile at a 2-fold level. 'No significant change' indicates genes that are expressed within the 2-fold level. The horizontal bars represent the gene number.



Figure 2. Gene expression profiles in UC patients, compared with normal subjects. The vertical bars indicate an increase or decrease in gene expression in patients with UC, compared with gene expression in control subjects, at a 2-fold level of the Cy5/Cy3 ratio of the UC sample divided by the Cy5/Cy3 ratio of the normal sample. 'Downregulated' designates genes that are downregulated, compared with the control profile, at a 2-fold level. 'No significant change' indicates genes that are expressed within the 2-fold level. (A) Expression profiles of 776 genes based on the JGS ID number. (B) Expression profiles of genes based on their functional classifications. The 776 genes were divided into 20 groups based on their functional relatedness: lymphokine/cytokine/chemokine (a, n=134), cell surface antigen/immune (b, n=65), kinase/kinase inhibitor (c, n=49), apoptosis/stress (d, n=37), matrix-related/membrane-bound (e, n=50), metal (f, n=24), oncogene/suppressor (g, n=65), cell cycle/transcription factor (h, n=42), proteasome (n, n=10), autoimmune (o, n=5), general enzyme (p, n=66), receptor (q, n=83), ribosomal protein (r, n=29), miscellaneous (s, n=57), and control (t, n=2). Since a single gene occasionally belongs to more than one group, the total number of genes indicated on the x-axis was 886.

contrast, none of the genes were downregulated by >2-fold in the UC patients. Fig. 2B shows the expression profiles of the genes based on their functional classifications. Compared with normal subjects, gene expression in a variety of functional groups was altered in patients with UC. Gene expression profiles: UC patients before LCAP vs. after LCAP. Fig. 3A shows the gene expression profiles of whole blood cells from UC patients before LCAP vs. those from the same patients after LCAP. The expression of several genes was altered following LCAP. Table IV displays several genes

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Table III. Ge	enes upregulated :	>2-fold in whole	blood cells of UC patien	ts vs. normal subjects.
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Gene	GenBank no.	Group	Mean
Galactoside-binding 3 (galectin 3); IgE-binding protein (ε-BP)	M57710	m	2.1
Immunoglobulin heavy constant $\gamma 3$	J00230	b	2.2
Calcium binding protein in macrophages (MRP14)	X06233	f	2.3
γ-G globin (HBG2)	X55656	j	2.5
Interleukin-1 receptor, type II (IL-1R2)	U74649	q	3.6

Genes are related to cell surface antigens/immune response (b), metals (f), energy/metabolism (j), serum protein/anti-coagulation (m), or receptors (q). The groups correspond to those in Fig. 2B.



Figure 3. Gene expression profiles in UC patients after LCAP, compared with those before LCAP. The vertical bars indicate an increase or decrease in gene expression in UC patients after LCAP, compared with gene expression in UC patients before LCAP, at a 2-fold level of the Cy5/Cy3 ratio of the UC sample divided by the Cy5/Cy3 ratio of the normal sample. 'Downregulated' designates genes that are downregulated, compared with the control profile, at a 2-fold level. 'No significant change' indicates genes that are upregulated, compared with the control profile, at a 2-fold level. 'No significant change' indicates genes that are expressed within the 2-fold level. (A) Expression profiles of 776 genes based on the JGS ID number. (B) Expression profiles of genes based on their functional classification. The 776 genes were divided into 20 groups based on their functional relatedness: lymphokine/cytokine/chemokine (a, n=134), cell surface antigen/immune (b, n=65), kinase/kinase inhibitor (c, n=49), apotsis/stress (d, n=37), matrix-related/membrane-bound (e, n=50), metal (f, n=24), oncogene/ suppressor (g, n=65), cell cycle/transcription factor (h, n=49), DNA/RNA-binding protein (i, n=42), energy/metabolism (j, n=38), drug metabolism (k, n=18), protease (1, n=21), serum protein/anti-coagulation (m, n=42), proteasome (n, n=10), autoimmune (o, n=5), general enzyme (p, n=66), receptor (q, n=83), ribosomal protein (r, n=29), miscellaneous (s, n=57), and control (t, n=2). Since a single gene occasionally belongs to more than one group, the total number of genes indicated on the x-axis was 886.

that were either upregulated or downregulated by >2-fold in UC patients who underwent LCAP. The downregulated transcripts included human leukocyte antigen (HLA)-DR β 1, HLA-DP light chain, CD74, CD97, and mangano-superoxide dismutase (Mn-SOD). In contrast, the upregulated transcripts included neutrophil gelatinase-associated lipocalin (NGAL), haptoglobin α 1S, α 1-acid glycoprotein, fos, matrix metalloproteinase 8 (MMP8), and the putative lymphocyte G0/ G1 switch gene. Fig. 3B shows the expression profiles of the genes based on their functional classification. As a result of LCAP, widespread functional groups of genes were either upregulated or downregulated.

Discussion

The development of microarray techniques has recently provided new tools capable of providing a more comprehensive image of the gene expression profiles underlying disease states. Using this innovative approach, we hoped to identify the cellular expression patterns of UC patients treated with LCAP. Circulating blood is composed of heterogeneous and changing cell populations. The interactions of immune cell populations with non-immune cellular components of the blood and, occasionally, precursor cells are thought to be pivotal to the pathophysiology of UC. Therefore, we chose to

Gene	GenBank no.	Group	Mean	SD
Downregulated genes				
Human leukocyte antigen (HLA)-DRβ1	M20430	b	0.4	0.1
Mangano-superoxide dismutase (Mn-SOD)	X14322	d	0.5	0.1
HLA-DP light chain	M57466	b	0.5	0.1
CD74	BC018726	b	0.5	0.1
CD97	U76764	b	0.5	0.1
Upregulated genes				
α-1 acid glycoprotein (orosomucoid-1)	M13692	m	2.5	1.0
Matrix metalloproteinase 8 (neutrophil collagenase)	J05556	f	3.9	2.7
Fos	BC004490	g	4.7	3.3
Haptoglobin α1S	X00637	m	5.0	3.4
Neutrophil gelatinase-associated lipocalin (NGAL)	X83006	g	5.2	3.3
Putative lymphocyte G0/G1 switch gene	M72885	h	6.5	4.5

Table IV. Genes upregulated or downregulated >2-fold in whole blood cells of pre-LCAP vs. post-LCAP UC patients.

Genes are related to cell surface antigens/immune response (b), apoptosis/stress (d), metals (f), oncogenes/suppressors (g), cell cycle/transcription factors (h), serum protein/anti-coagulation (m), or miscellaneous factors (s). The groups correspond to those in Fig. 3B.

use RNA from whole blood cells, which comprises heterogeneous cell types, with the specific purpose of gaining a global and representative insight into all cellular changes associated with LCAP. Furthermore, we used freshly obtained peripheral blood samples, and the RNA was immediately stabilized in Pax-Gene tubes. This protocol is critical because it has been previously shown that even short-term *ex vivo* incubations of blood cells can alter expression profiles (27).

We first compared the gene expression profiles of whole blood cells from UC patients with those from normal subjects. Our results identified several upregulated genes including IL-1R2, an IL-1R that antagonizes IL-1-mediated events (28), and MRP-14, a calcium-binding protein expressed during chronic inflammation (29). Several genes that have not been previously linked to UC, such as HBG2 (30), galectin 3 (31), and immunoglobulin heavy constant γ 3 (32), were also upregulated. The widespread upregulation of these genes involved in receptor (IL-1R2), metal (MRP-14), energy/metabolism (HBG2), serum protein/anti-coagulation (galectin 3), and cell surface antigen/immune (immunoglobulin heavy constant y3) processes may indicate a major disruption in cellular homeostasis in UC. Further studies are needed to elucidate whether the gene expression profile observed in this study is specific to UC or merely a secondary event associated with intestinal inflammation.

LCAP is a therapeutic strategy involving extracorporeal immunomodulation that has been used to treat several immunological disorders including UC (8-11) and Crohn's disease (12). A multicenter, randomized controlled trial of UC patients showed that the efficacy of LCAP was significantly superior to that of high-dose steroid therapy (9). Recent research has revealed that LCAP preferentially attenuates inflammatory and immune responses through the downregulation of proinflammatory mediators, such as IL-1, tumor necrosis factor- α or adhesion molecules, or the upregulation of anti-inflammatory mediators, such as IL-4 or IL-10 (33-35). In the present study,

we focused on, for the first time, the effect of LCAP on the gene expression profiles of whole blood cells from UC patients.

LCAP downregulated the expression of proinflammatory genes that belong to the cell surface antigen/immune response group, including CD97, CD74, HLA-DRβ1 and HLA-DP light chain. CD97 has an essential role in the migration of neutrophils by facilitating the binding of chemokines (36). In fact, the homing of adoptively transferred neutrophils to the colon was delayed when the cells were preincubated with anti-CD97 monoclonal antibodies in experimental colitis (37). CD74, a cell surface binding protein for macrophage migration inhibitory factor (MIF), is required for the MIF-induced activation of the extracellular signal-regulated kinase-1/2 kinase cascade, cell proliferation, and prostaglandin E2 production (38). Moreover, MIF, the ligand for CD74, plays a key role in the development of IBD (39). The downregulation of HLA-related genes, such as HLA-DRB1 and HLA-DP light chain, may imply an improvement in the abnormal immune regulation observed in UC (40).

Notably, LCAP upregulated the expression of natural antimicrobial NGAL, a neutrophil lipocalin that may bind the proinflammatory bacterial tripeptide N-formylmethionylleucyl-phenylalanine (41). In situ hybridization and immunohistochemical studies have shown strong NGAL expression in colonocytes and neutrophils in patients with UC (42). Since enteric flora plays an important role in the pathogenesis of UC, the upregulation of such genes lends particularly strong support to the effectiveness of LCAP. Moreover, several acute phase protein genes, including haptoglobin α 1S and α 1-acid glycoprotein, were upregulated after LCAP. Previous studies have shown that haptoglobin, found in chronic inflammatory conditions, can aid in tissue repair by stimulating angiogenesis (43). Furthermore, α 1-acid glycoprotein can prevent neutrophil activation during inflammatory processes (44). Therefore, the LCAP-induced upregulation of these three genes seems to play a protective role in patients with UC. The functional roles of the upregulation of Mn-SOD (45), the putative lymphocyte G0/G1 switch gene (46), fos (47) and MMP8 (48) remain unclear.

How does LCAP alter these cellular gene expression profiles? During LCAP, most of the leukocytes are removed by a filter during extracorporeal circulation. This treatment is associated with rebound leukophilia in response to transient leukopenia (49,50), indicating a rapid cell release response from the reticuloendothelial system. Therefore, this drastic change in gene expression may be partly explained by the replacement of activated leukocytes with new and naïve ones originating from the marginated pool and/or bone marrow. We cannot completely exclude the possibility that some of the identified differences in gene expression before and after LCAP may be the result of a distinct composition of leukocyte subsets, since the proportion of neutrophils increased and the proportion of lymphocytes and monocytes decreased after LCAP.

Medical therapies for IBD are predominantly directed at attenuating inflammatory and immune processes using glucocorticoids, immunosuppressants, and/or cytokine inhibitors (51,52). New therapeutic approaches that may change the enteric flora, promote tissue repair, or enhance the antiinflammatory aspects of the disease have also been employed (53,54). Since the analyses in the present study were conducted using only six samples obtained from patients after their first session of LCAP, we could not clarify whether the change in the cellular gene expression profiles directly contributed to the positive clinical outcomes after a full course of LCAP. However, taking into consideration the evidence presented here, we speculate that LCAP preferentially downregulates genes related to disease progression and upregulates those related to disease amelioration.

In conclusion, we identified, for the first time, the gene expression profile of whole blood cells from patients with UC and the transcriptional events following LCAP. After LCAP, the gene expression profile shifted toward one indicating disease improvement. These results suggest a basis for the molecular mechanisms leading to the therapeutic effects of LCAP as well as indicating new therapeutic targets, thereby providing important prognostic information.

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