# Gene Expression Signature and the Prediction of Ulcerative Colitis–Associated Colorectal Cancer by DNA Microarray

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**Abstract**

**Purpose:** Ulcerative colitis (UC) is associated with a high risk of colorectal cancer. To identify genes that could predict the development of cancer in UC, we conducted a DNA microarray analysis using nonneoplastic rectal mucosa of UC patients.

**Experimental Design:** Gene expression in nonneoplastic mucosa of 53 UC patients were examined. Gene expression profiles were examined using human Genome U133 Plus 2.0 gene chip array (Affymetrix). Among 53 UC patients, 10 had UC-associated cancer (UC-Ca group) whereas 43 did not (UC-NonCa group).

**Results:** By comparing gene expression profiles of nonneoplastic rectal mucosae between the UC-Ca and UC-NonCa groups, we could identify 40 genes that were differentially expressed between two groups. The list of discriminating genes included low-density lipoprotein receptor–related protein (*LRP5* and *LRP6*). Previous studies suggested that LRP5 and LRP6 expression promotes cancer cell proliferation and tumorigenesis and are considered as candidate oncogenes. In the present study, both LRP5 and LRP6 showed significantly higher expression in the UC-Ca group, which suggests the importance of these genes in the development of UC-associated colorectal cancers. With the 40 selected discriminating genes, we did class prediction of the development of colorectal neoplasms in UC patients. Using the *k*-nearest neighbor method and the support vector machine, we could predict the development of UC-associated neoplasms with an accuracy of 86.8% and 98.1%, respectively.

**Conclusions:** These findings have important implications for the early detection of malignant lesions in UC and may provide directions for future research into the molecular mechanisms of UC-associated cancer.

Ulcerative colitis (UC) is a chronic inflammatory disease associated with a high risk of colon cancer. UC-associated colon cancer has been reported to develop in a different way from sporadic colon cancer, which is sometimes called "inflammation dysplasia carcinoma sequence" (1–3). Neoplastic lesions in UC are divided into dysplasia and cancer (4, 5). Dysplasia is often seen in patients with UC-associated cancer and considered to be a precursor of cancer (4, 5). The cumulative risk of developing UC-associated colon cancer increases with the duration and extent of the disease. At the present time, patients with total colitis with the disease lasting >7 years are considered to be at risk of cancer and, therefore, surveillance colonoscopy is done on such patients (6, 7). However, if we can further select patients at a higher risk of cancer, more effective and intensive follow-up can be done. Therefore, new markers that predict the development of cancer or dysplasia in UC are urgently needed, allowing for more selective treatment strategies for higher-risk patients. Previous studies have shown that patients with UC-associated colon cancer and/or dysplasia have widespread genetic alterations in nonneoplastic colonic mucosa (8–12). These results suggested the possibility that changes in nonneoplastic mucosa might be molecular markers for predicting the development of cancer and/or dysplasia in UC. However, subsequent studies have not confirmed these results, and, to date, no specific molecular markers have been established. On the other hand, recent advances in DNA microarray technology have shown the potential use of expression profiles for molecular classification of cancer, as

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well as for prediction of disease outcome (13–16). However, no study has pointed to the possibility of gene expression profiles in predicting the development of cancer and/or dysplasia in UC. Recently, we reported that gene expression profiles could predict response to radiotherapy in rectal cancer (17). Applying the same method in the present study, we examined expression profiles in nonneoplastic rectal mucosa in UC patients. Our objective here was to identify a set of genes of which the expression profiles can be used to predict the development of cancer and/or dysplasia in UC.

## Materials and Methods

***Patients and samples.*** Fifty-three UC patients were examined. 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 >7 years; they were therefore considered to be at high risk of cancer and/or dysplasia. Among 53 UC patients, 10 had UC-associated neoplastic lesions, including 8 adenocarcinoma and 2 dysplasia (UC-Ca group). All lesions were localized in the large bowel and no case showed metastatic disease in cases with cancer. Among the cases of dysplasia, one case showed low-grade dysplasia in the sigmoid and the transverse colon, whereas another showed low-grade dysplasia in the sigmoid colon. Forty-three UC patients had no neoplastic lesions (UC-NonCa group). Between the UC-Ca group and the UC-NonCa group, there were no significant differences in age at biopsy, age at UC diagnosis, gender, duration of disease, extent of UC, presence of inflammation medication, presence of primary sclerosing cholangitis, and disease activity (Supplementary Table S1). In all UC cases, specimens were obtained from nonneoplastic rectal mucosa for RNA extraction (Fig. 1). Samples were taken either from surgically resected specimens or during surveillance colonoscopic examination. Surveillance was done, preferentially avoiding periods of clinical relapse, and specimens were histologically evaluated as recommended by the practice guidelines (18). We excluded patients who underwent surveillance during the relapse phase. From surgically resected specimens, mucosal samples were obtained and processed so that they would be equivalent to the endoscopic biopsies. Samples were snap-frozen in liquid nitrogen and stored at −80°C until use. Paralleled tumor specimens were formalin fixed and paraffin embedded for histologic examination. Samples were used for RNA extraction when microscopic examination verified that no neoplastic cells were present.

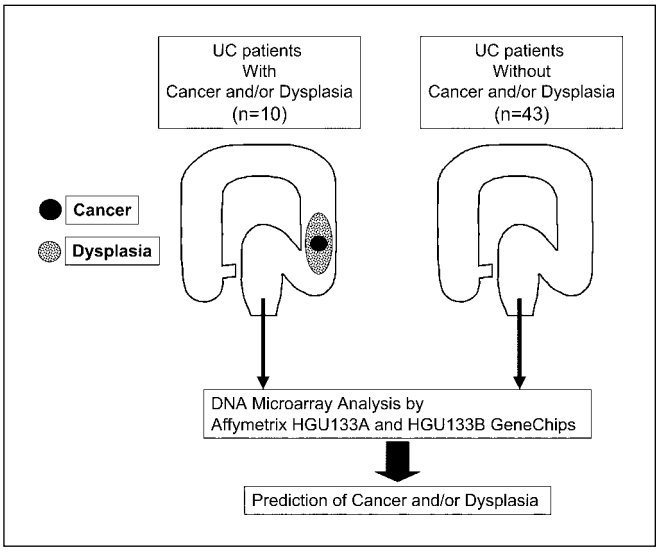


**Fig. 1.** Sample collection and data analysis procedure. Samples were obtained from nonneoplastic rectal mucosa for RNA extraction. Gene expression profiles were determined using Affymetrix HGU133A and HGU133B GeneChips (Affymetrix) and class prediction was done.

***RNA isolation and microarray procedures.*** Total RNA was isolated from each of the frozen samples with RNeasy Mini Kit (Qiagen, Chatswort, CA) for gene expression analysis. Gene expression profiles were determined using Affymetrix HGU133A and HGU133B GeneChips (Affymetrix, Santa Clara, CA) according to the manufacturer's recommendations. In brief, double-stranded cDNA was synthesized with 8 μg of total RNA with oligo $d(T)_{24}$ T7 primer and transcribed into biotinylated cRNA using the IVT Labeling Kit (Affymetrix). Twenty micrograms of the biotinylated cRNA were fragmented at 94°C for 35 min and hybridized to human Genome U133 Plus 2.0 gene chip array (Affymetrix), which contains >54,000 probe sets. The hybridized cRNA probes to oligonucleotide arrays were stained with streptavidin R-phycoerythrin and were scanned using GeneChip Scanner 3000 (Affymetrix). The scanned data were processed for signal values using Micro Array Suite 5.0 software (Affymetrix). All data used for subsequent analysis passed the quality control criteria.[7]

***Statistical analysis.*** Gene expression profiles of nonneoplastic rectal mucosa (UC-Ca and UC-NonCa groups) and cancer (UC-Ca group) were carried out with GeneSpring software version 7.2 (Silicongenetics, Redwood, CA). Gene expression data, when classified as either flag-P or flag-M in >30% of all samples, were loaded into the software. All expression data on a chip were normalized to the 50th percentile of all values on that chip followed by normalization to the median expression level of that gene across all samples.

***Class comparison and prediction between the UC-Ca and UC-NonCa groups.*** To determine the discriminating genes between the UC-Ca and UC-NonCa groups, the data sets of nonneoplastic rectal mucosa in UC were assigned to either the UC-Ca group or the UC-NonCa group. The expression profiles of the two groups were then compared using unpaired *t* tests (with Welch's correction for unequal variances) and a fold-change value to identify genes that were differentially expressed between them. Two-dimensional hierarchical clustering was then applied to the log-transformed data with average-linkage clustering, with standard correlation as the similarity metric for the discriminating genes that we identified as differentially expressed between the UC-Ca and UC-NonCa groups. Variation in multigene expression between the two groups was compared by principal component analysis. We carried out supervised class prediction with the *k*-nearest neighbor method and the support vector machine and a leave-one-out cross-validation with selected discriminating genes (19, 20).

***Gene functional category analysis.*** Gene Ontology categories were analyzed by the BioScript Library tool in GeneSpring 7.2. Genes were classified according to their annotated role in biological processes, molecular function, and cellular components from Gene Ontology (The Gene Ontology Consortium). A hypergeometric *P* value was used to measure statistical significance of the overlap (i.e., the likelihood that it is a coincidence that these many genes were in both the experimentally extracted gene list and the category).

## Results

***Gene expression profiling: class comparison between the UC-Ca and UC-NonCa groups.*** Gene expression profiling of rectal mucosa was established using DNA array in all samples. To identify molecular signatures that may characterize the development of UC-associated neoplasm, gene expression profiles in the UC-Ca and UC-NonCa groups were compared. We were

[7] The entire microarray data set is available at http://www.ncbi.nlm.nih.gov/geo/info/linking.html under the data series accession no. GSE3629.

**Table 1.** List of 40 discriminating genes between the UC-Ca and UC-NonCa groups

| Probe ID | Fold change (UC-Ca group/ UC-Non Ca group) | *P* | Gene symbol | GenBank | Gene name |
|---|---|---|---|---|---|
| 231341_at | 4.785 | 0.0047 | | BE670584 | *Homo sapiens* transcribed sequences |
| 215807_s_at | 2.695 | 0.0106 | *PLXNB1* | AV693216 | plexin B1 |
| 205330_at | 2.604 | 0.0496 | *MN1* | NM_002430 | meningioma (disrupted in balanced translocation) 1 |
| 230465_at | 2.577 | 0.0232 | *HS2ST1* | AI806674 | heparan sulfate 2-*O*-sulfotransferase 1 |
| 213182_x_at | 2.488 | 0.0047 | *CDKN1C* | R78668 | cyclin-dependent kinase inhibitor 1C (p57, Kip2) |
| 228441_s_at | 2.439 | 0.0204 | *PET112L* | BE550153 | *Homo sapiens* transcribed sequences |
| 219534_x_at | 2.049 | 0.0463 | *CDKN1C* | NM_000076 | cyclin-dependent kinase inhibitor 1C (p57, Kip2) |
| 229953_x_at | 1.946 | 0.0454 | *C6orf152* | AI742190 | chromosome 6 open reading frame 152 |
| 212327_at | 1.942 | 0.0399 | *KIAA1102* | AK026815 | KIAA1102 protein |
| 219514_at | 1.938 | 0.0242 | *ANGPTL2* | NM_012098 | angiopoietin-like 2 |
| 235318_at | 1.898 | 0.0496 | | AW955612 | *Homo sapiens* transcribed sequences |
| 209468_at | 1.842 | 0.0444 | *LRP5* | AB017498 | low-density lipoprotein receptor–related protein 5 |
| 211740_at | 1.828 | 0.0475 | *ICA1* | BC005922 | islet cell autoantigen 1, 69 kDa |
| 213652_at | 1.730 | 0.0444 | *PCSK5* | AU152579 | Proprotein convertase subtilisin/kexin type 5 |
| 201194_at | 1.715 | 0.0481 | *SEPW1* | NM_003009 | selenoprotein W, 1 |
| 225275_at | 1.664 | 0.0481 | *EDIL3* | AA053711 | epidermal growth factor–like repeats and discoidin I-like domains 3 |
| 59631_at | 1.639 | 0.0242 | | 4854760_RC | Cluster Incl. AI247566:qh60e06.x1 |
| 221566_s_at | 1.626 | 0.0246 | *NOL3* | AF043244 | nucleolar protein 3 (apoptosis repressor with CARD domain) |
| 225745_at | 1.558 | 0.0087 | *LRP6* | AV725248 | low-density lipoprotein receptor–related protein 6 |
| 202786_at | 1.508 | 0.0444 | *STK39* | NM_013233 | serine threonine kinase 39 |
| 209770_at | 0.661 | 0.0357 | *BTN3A1* | U90552 | butyrophilin, subfamily 3, member A1 |
| 226153_s_at | 0.640 | 0.0078 | *CNOT6L* | AW514857 | CCR4-NOT transcription complex |
| 224909_s_at | 0.619 | 0.0173 | *PREX1* | BF308645 | phosphatidylinositol, RAC exchanger 1 |
| 235175_at | 0.617 | 0.0047 | *GBP4* | BG260886 | guanylate binding protein 4 |
| 202269_x_at | 0.616 | 0.0322 | *GBP1* | BC002666 | guanylate binding protein 1, IFN-inducible |
| 205270_s_at | 0.596 | 0.0087 | *LCP2* | NM_005565 | lymphocyte cytosolic protein 2 |
| 226080_at | 0.595 | 0.0100 | *SSH2* | BE676214 | slingshot 2 |
| 231747_at | 0.564 | 0.0444 | *CYSLTR1* | NM_006639 | cysteinyl leukotriene receptor 1 |
| 206974_at | 0.552 | 0.0082 | *CXCR6* | NM_006564 | chemokine (C-X-C motif) receptor 6 |
| 231577_s_at | 0.548 | 0.0444 | *GBP1* | AW014593 | guanylate binding protein 1 |
| 226474_at | 0.509 | 0.0206 | *NOD27* | AA005023 | nucleotide-binding oligomerization domains 27 |
| 229625_at | 0.482 | 0.0203 | *GBP5* | BG545653 | guanylate binding protein 5 |
| 217629_at | 0.460 | 0.0342 | | AA365670 | transcribed sequence with similarity to eIEF associated HSPC021 |
| 1555638_a_at | 0.456 | 0.0292 | *SAMSN1* | AF519621 | SAM domain, SH3 domain and nuclear localisation signals, 1 |
| 1560706_at | 0.452 | 0.0302 | | AL832268 | *Homo sapiens* mRNA; cDNA DKFZp667N1617 |
| 239946_at | 0.452 | 0.0394 | | AA776723 | *Homo sapiens* transcribed sequences |
| 238581_at | 0.431 | 0.0350 | *GBP5* | BG271923 | guanylate binding protein 5 |
| 220330_s_at | 0.431 | 0.0297 | *SAMSN1* | NM_022136 | SAM domain, SH3 domain and nuclear localisation signals, 1 |
| 203760_s_at | 0.310 | 0.0444 | *SLA* | U44403 | Src-like-adaptor |
| 203819_s_at | 0.288 | 0.0206 | *IMP-3* | AU160004 | insulin-like growth factor-II mRNA-binding protein 3 |

able to identify 40 genes (*t* test with false discovery rate, $P < 0.05$), which were differentially expressed between the two groups (Table 1). Twenty genes showed higher expression and 20 genes lower expression in the UC-Ca group than in the UC-NonCa group. The list of genes included an apoptosis repressor (*NOL3*), cyclin-dependent kinase inhibitor (*CDKN1C* and *p57*), insulin-like growth factor-II mRNA-binding protein (*IMP-3*), and other genes related to cell proliferation and/or signal transduction (*LRP5/6* and *PLXNB1*). Results of a hierarchical cluster analysis of the 40 genes are presented in Fig. 2. Patients in the UC-Ca and UC-NonCa groups were clustered into two distinct groups. All patients were correctly classified into either the UC-Ca group or the UC-NonCa group except for three cases in the UC-NonCa group. We then used the 40 discriminating genes to generate a three-dimensional plot (from a 40-dimensional plot) of the data (Fig. 3). Principal component analysis–based multidimensional scaling visualization separated samples in the UC-CA and UC-NonCa groups into a linearly separable gene expression data space.

***Gene functional category analysis.*** To investigate the biological functions involved in the discriminating genes, we did

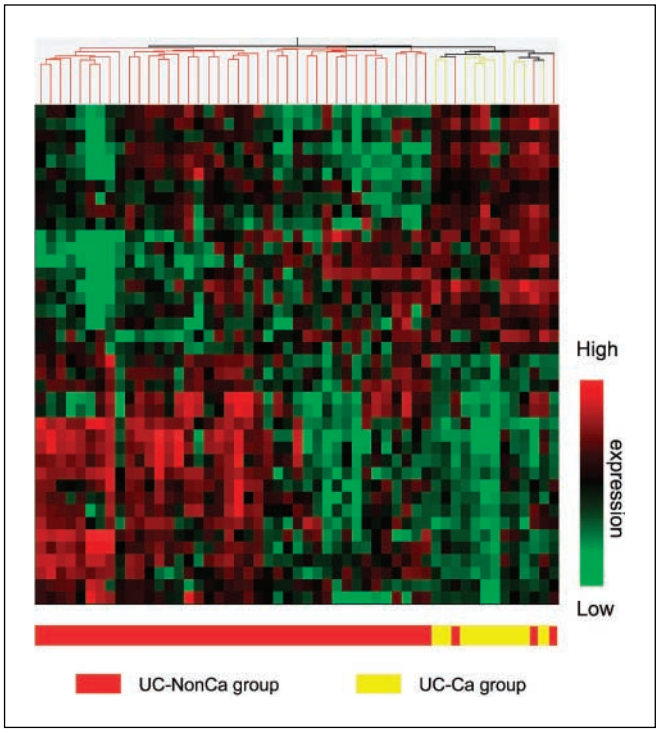


**Fig. 2.** Two-way hierarchical clustering was used to order samples (*columns*) and array targets (*rows*). Red, overexpression; green, underexpression. Patients in the UC-Ca and UC-NonCa groups were clustered into two distinct groups. All patients were classified correctly into either the UC-Ca group or the UC-NonCa group except for three cases in the UC-NonCa group. Bottom, yellow, UC-Ca group; red, UC-NonCa group.

Gene Ontology category analysis. Forty genes were categorized to a variety of biological process categories. Categories such as receptor activity, signal transduction, regulation of gene expression, cell-cell signaling, cell cycle, cell proliferation, and protein kinase activity showed higher percentage as compared with other categories (Fig. 4). When expressions of the 40 discriminating genes were compared with those of other genes of which the expression profiles could be evaluated, some categories, such as signal transduction, receptor activity, receptor binding, and lipid metabolism, showed a significantly higher proportion among 40 selected genes (Fig. 4).

***Class prediction of development of UC-associated neoplasm.*** We next examined whether the expression profiling is useful in predicting the development of UC-associated neoplasm. Using the 40 discriminating genes, supervised class prediction of the UC-Ca and UC-NonCa groups was done with the *k*-nearest neighbor method and the support vector machine with leave-one-out cross-validation (19, 20). We were able to predict the development of UC-associated neoplasm with an accuracy of 86.8% by *k*-nearest neighbor method and 98.1% by support vector machine. Furthermore, we evaluated the sensitivity, specificity, positive predictive value, and negative predictive value of the predictive model. By *k*-nearest neighbor method, the sensitivity, specificity, positive predictive value, and negative predictive value were 100.0%, 83.7%, 58.8%, and 100.0%, respectively. There were seven misclassifiers, all in the UC-NonCa group (Supplementary Fig. S1). On the other hand, by support vector machine, the sensitivity, specificity, positive predictive value, and negative predictive value were 100.0%, 97.6%, 90.9%, and 100.0%, respectively. There was only one misclassifier in the UC-NonCa group.

## Discussion

By using expression profiling of nonneoplastic rectal mucosa, we were able to identify patients with UC-associated neoplasm with a high accuracy rate. In UC carcinogenesis, it is suggested that there is a field effect of chronic inflammation with the necessity for constantly increased reepithelialization and suppression of cell cycle–regulating proteins, which may make genes susceptible to genetic damage. In fact, previous studies have shown that specific gene alteration is already present in nonneoplastic mucosa in UC patients with dysplasia

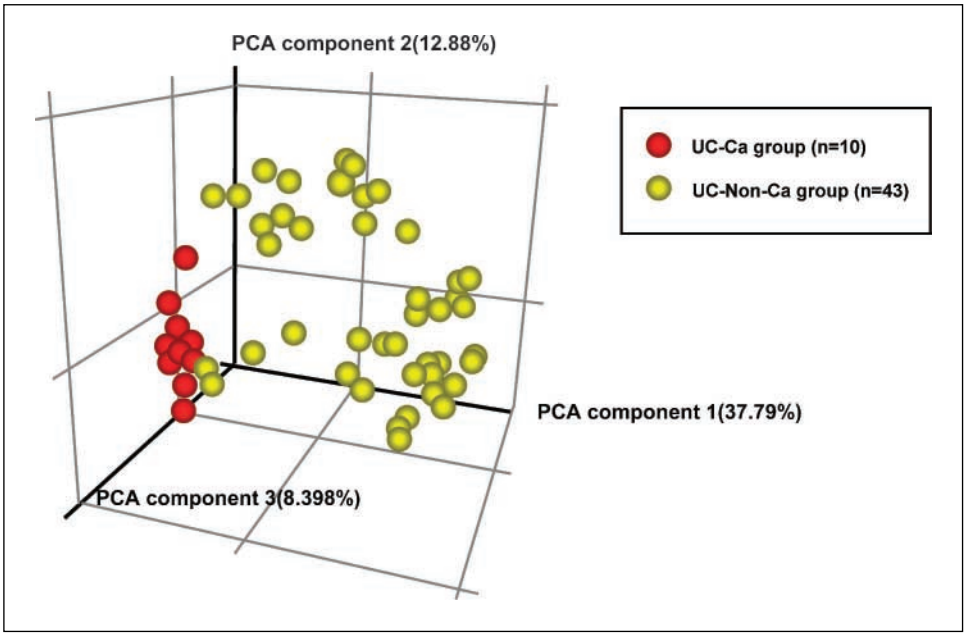


**Fig. 3.** Principal component analysis. Discriminating genes were used to generate a three-dimensional plot (from a 40-dimensional plot) of the data. Principal component analysis (PCA)–based multidimensional scaling visualization separated samples in the UC-Ca (*red*) and UC-NonCa (*yellow*) groups into linearly separable gene expression data space.

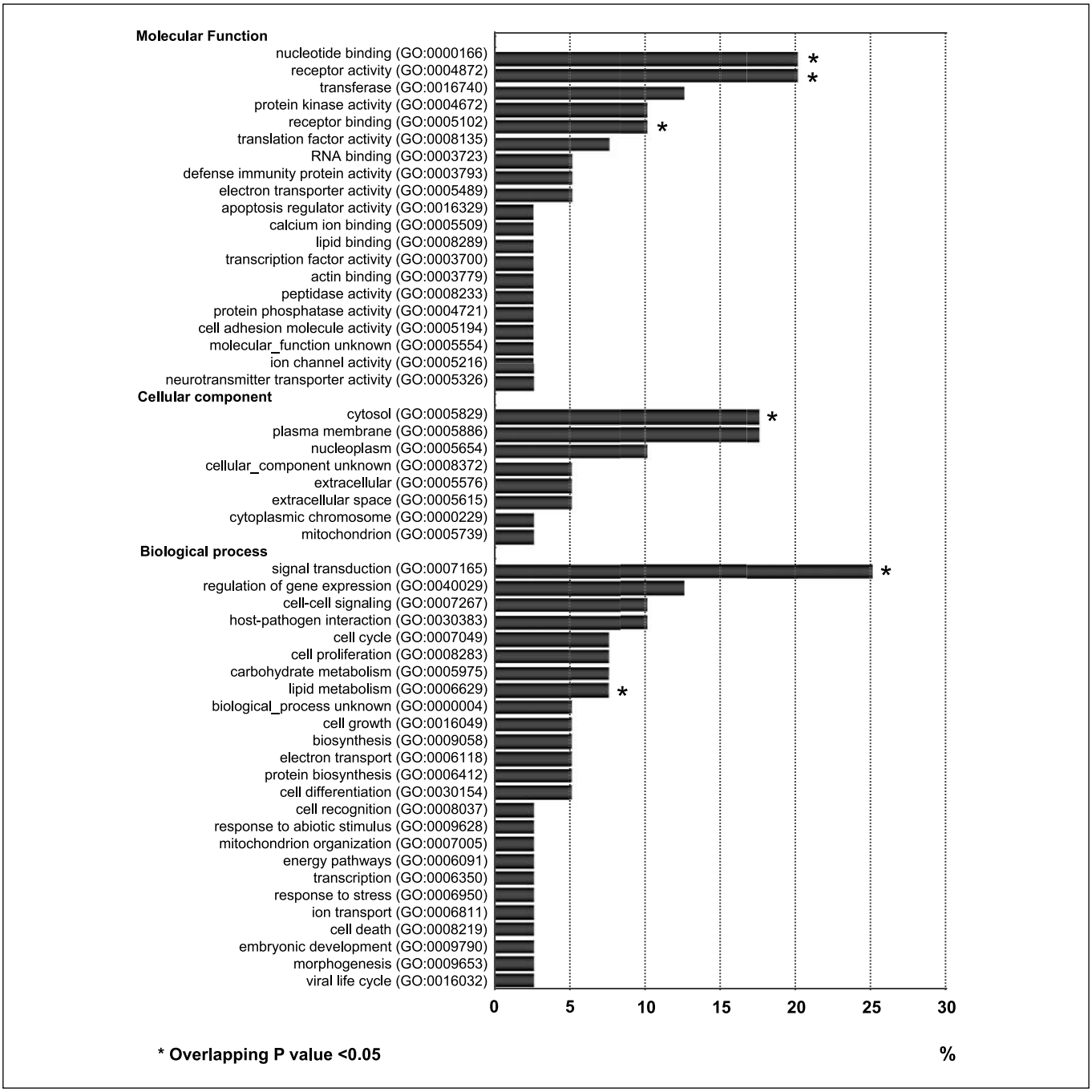


**Fig. 4.** Gene Ontology analysis of 40 discriminating genes. Categories of receptor activity, signal transduction, and cell proliferation showed a higher percentage as compared with other categories. Some categories, such as signal transduction and receptor activity, showed a significantly higher proportion among selected 40 genes (overlapping *P* value, <0.05).

and/or cancer (8–12). These genes include *hMLH1*, *p16*, *p14*, and *ER*. Therefore, it has been suggested that these changes may be biomarkers for the early detection of cancer or dysplasia. However, this has not been confirmed by subsequent studies or has become routinely used in the clinical setting. Furthermore, each of these studies has examined only a single or a few genes to predict development of cancer. In the present study, we used microarray analysis for selection of patients with UC-associated neoplasm. The present study first showed that microarray analysis is useful for predicting the development of UC-associated neoplasm with a high accuracy rate.

By examining >50,000 genes and transcripts, we were able to select the 40 discriminating genes of which the expression differed significantly in the UC-Ca and UC-NonCa groups. To exclude the affects of clinical characteristics in selecting discriminating genes, we confirmed that there was no significant difference in characteristics such as presence of inflammation, duration of disease, age, etc., between the UC-Ca and UC-NonCa group. Using 40 genes, the two-way hierarchical

clustering and principal component analysis could distinguish the UC-Ca and UC-NonCa groups. Furthermore, we did class prediction of the UC-Ca and UC-NonCa groups. The accuracy rates of supervised class prediction with the 40 discriminating genes by *k*-nearest neighbor method and support vector machine were 86.8% and 98.1%, respectively. The sensitivity and specificity of the present model were 100% and 83.7% by *k*-nearest neighbor method and 100% and 97.6% by support vector machine, respectively. Furthermore, considering that negative predictive value was 100.0% by both *k*-nearest neighbor method and support vector machine, this model seems to enable to select "low-risk" patients for developing cancer in UC. These results suggested the possibility that the present model may be used for early detection of UC-associated neoplasm. However, one limitation of the present study was the small number of patients with UC-associated neoplasm (10 patients). Therefore, to use the present model in a clinical setting, we believe that we need to validate the accuracy of the present model in a new independent set of samples from a larger number of patients.

Among the 40 discriminating genes, genes showing higher expression in the UC-Ca group than in the UC-NonCa group included cyclin-dependent kinase inhibitor (*CDKN1C*), serine threonine kinase (*STK39*), apoptosis inhibitor (*NOL3*), and low-density lipoprotein receptor–related protein (*LRP5* and *LRP6*). Gene function analysis revealed that categories, such as signal transduction, receptor activity, receptor binding, and lipid metabolism, showed a significantly higher proportion among 40 selected genes when compared with other genes of which the expression profile could be evaluated. It was suggested that genes in these categories might be involved in determining the development of UC-associated neoplasm. Especially, among genes related to signal transduction, we could identify *LRP5* and *LRP6* as the discriminating genes. LRP5 and LRP6 are components of receptors for the Wnt pathway and transduce an intracellular Wnt signal (21, 22). Recent studies suggested that *LRP5* and *LRP6* are candidate oncogenes and expression of LRP5 is associated with metastatic disease in human osteosarcoma (21). At the present time, the precise role of LRP5 and LRP6 in the development of colorectal cancer remains unknown. However, it has been well known that the Wnt pathway plays an important role in colorectal carcinogenesis and the discriminating genes included not only *LRP5* but also *LRP6*. Furthermore, the average expression level of both *LRP5* and *LRP6* was higher in cases with cancer than in those with dysplasia (data not shown). These results suggested that *LRP5* and *LRP6* might play a significant role in the development of UC-associated cancers.

In conclusion, we identified a gene expression signature in nonneoplastic rectal mucosa in UC patients, which is predictive of the development of UC-associated neoplasm. The choice of how aggressively the patient should be treated may be determined by the predicted outcome of the present model. Intensive care for patients at higher risk may make it possible to detect UC-associated neoplasm earlier and more effectively. However, because the number of patients, especially those with UC-associated neoplasm, was limited in the present study, large prospective trials will be needed to confirm the validity of the present predictor. Furthermore, biological gene categories enriched in patients with UC-associated neoplasm may provide directions for future research into the molecular mechanisms of UC-associated cancer.



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