

# Inflammation, adenoma and cancer: Objective classification of colon biopsy specimens with gene expression signature

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**Abstract.** Gene expression analysis of colon biopsies using high-density oligonucleotide microarrays can contribute to the understanding of local pathophysiological alterations and to functional classification of adenoma (15 samples), colorectal carcinomas (CRC) (15) and inflammatory bowel diseases (IBD) (14). Total RNA was extracted, amplified and biotinylated from frozen colonic biopsies. Genome-wide gene expression profile was evaluated by HGU133plus2 microarrays and verified by RT-PCR. We applied two independent methods for data normalization and used PAM for feature selection. Leave one-out stepwise discriminant analysis was performed. Top validated genes included collagenIV $\alpha$ 1, lipocalin-2, calumenin, aquaporin-8 genes in CRC; CD44, met proto-oncogene, chemokine ligand-12, ADAM-like decysin-1 and ATP-binding cassette-A8 genes in adenoma; and lipocalin-2, ubiquitin D and IFITM2 genes in IBD. Best differentiating markers between Ulcerative colitis and Crohn's disease were cyclin-G2; tripartite motif-containing-31; TNFR shedding aminopeptidase regulator-1 and AMICA. The discriminant analysis was able to classify the samples in overall 96.2% using 7 discriminatory genes (indoleamine-pyrrole-2,3-dioxygenase, ectodermal-neural cortex, TIMP3, fucosyltransferase-8, collectin sub-family member 12, carboxypeptidase D, and transglutaminase-2). Using routine biopsy samples we successfully performed whole genomic microarray analysis to identify discriminative signatures. Our results provide further insight into the pathophysiological background of colonic diseases. The results set up data warehouse which can be mined further.

**Keywords:** Gene expression signature, whole genomic oligonucleotide microarray, colon cancer, adenoma, inflammatory bowel disease

## 1. Introduction

Colorectal cancer (CRC) is one of the most frequent death-causing tumorous diseases in Western countries. CRC frequently follows various high-risk conditions

such as adenomatous polyps and inflammatory bowel disease (IBD). The exact diagnosis of IBD types is still often difficult by conventional histology. High-density oligonucleotide microarray analysis gives an opportunity for studying the genetic and gene expression background, understanding of local pathophysiological alterations and for functional classification of diseases.

To date, microarray analyses reported in the literature were performed predominantly from surgically removed CRC samples [16], while microarray gene expression profiling of adenomas and IBDs as colorectal

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diseases predisposing to CRC has been studied less. Besides the publications dealing with the comparison of gene expression profiles (GEP) of CRC and normal colonic mucosa [10,21,29], more and more scientific studies appear to focus on gene expression background of CRC progression and metastases development [1–4, 22,25,26,42,43], characterization of CRC subtypes according to mRNA expression [3,14,44], the correlation of GEP with clinicopathological parameters [3,5,7,44], and on the generation of mRNA expression based prognosis [40]. Microarray-based molecular diagnostics of malignancy in colon adenoma and CRC samples were described using 10 [1], 9 [25,26] and 4 [29] adenoma samples compared to adenocarcinoma and normal colonic tissues. Expression microarray analyses of IBD samples were performed in order to determine global GEP of mucosal samples from patients with IBD compared to normal controls [23], to identify novel candidates for ulcerative colitis (UC) and/or Crohn's disease (CD) genetic susceptibility [24,30], to find marker genes involved in IBD-related carcinogenesis [38], to compare expression of entire chemokine family within IBD and normal patients [31], and to examine changes in GEP in peripheral blood mononuclear cells in IBD patients [27].

mRNA expression array analysis is usually performed using high volume surgically removed tissues. In the gastrointestinal tract, biopsy samples are routinely taken during the endoscopic examination with minimal intervention. The mRNA expression study of these samples could allow further insight into the development of inflammatory, preneoplastic and neoplastic diseases, and these biopsy specimens could be suitable samples for identifying early diagnostic target molecules. Colonic biopsies were applied previously for expression array analysis only in several cases [15, 23,30,31], because, even today, array technology needs significantly more RNA than can be isolated from tiny biopsy specimens. However, new techniques and commercial kits have recently become available for the reliable mRNA amplification without any effect on the original gene expression pattern [39].

Genome-wide gene expression profiling studies using microarrays have the potential to improve the diagnosis and treatment of human cancers and other disorders. However, recently appeared whole genomic oligonucleotide microarrays representing more than 47000 transcripts have not been used in any type of gastrointestinal disorders.

In the present study we aimed to find discriminatory genes between the main diagnostic groups and to

develop and test validation assay system and to confirm the applicability of biopsy samples for microarray analysis-based classification. Another purpose of our work was to search for altered biological pathways for explanation of the pathomechanism of these colonic diseases based on whole genomic mRNA microarray results.

## 2. Materials and methods

### 2.1. Patients and samples

After informed consent, biopsy samples were taken from the colon during the endoscopic intervention before treatment, and stored in RNALater Reagent (Qiagen Inc., US) at  $-80^{\circ}\text{C}$ . Total RNA was extracted from biopsies of 15 patients with tubulovillous/villous adenomas, 15 with colorectal adenocarcinoma (all microsatellite negative), 9 with active ulcerative colitis (UC), 5 with active Crohn's colitis (CD) and of 8 healthy normal controls (Table 1). Detailed patient specification is described in Supplemental Table 1.

### 2.2. Methods

#### 2.2.1. Microarray analysis

Total RNA was extracted using RNeasy Mini Kit (Qiagen, US) according to the manufacturer's instructions. Quantity and quality of the isolated RNA were tested by measuring of the absorbance and agarose gelelectrophoresis. Biotinylated cRNA probes were synthesized from 5–8  $\mu\text{g}$  total RNA and fragmented according to the Affymetrix description using GeneChip cDNA synthesis reagents and sample cleanup module and Enzo BioArray HighYield RNA Transcript Labeling Kit ([https://www.affymetrix.com/support/downloads/manuals/expression\\_s2\\_manual.pdf](https://www.affymetrix.com/support/downloads/manuals/expression_s2_manual.pdf) – first version). 10  $\mu\text{g}$  of each fragmented cRNA sample was hybridized into HGU133 Plus2.0 array (Affymetrix Inc.) at  $45^{\circ}\text{C}$  for 16 hours. The slides were washed and stained using Fluidics Station 450 and antibody amplification staining method according to the manufacturer's instructions. The fluorescent signals were detected by a GeneChip Scanner 3000.

Table 1  
Patient groups investigated in our study

Group	n	Male/female	Age	Taqman available
Adenoma without dysplasia	6	3/3	65.2 ± 15.9	6
Adenoma with dysplasia	9	3/6	73.6 ± 11.6	6
Ulcerative colitis	9	3/6	43.8 ± 15.9	7
Crohn's disease	5	2/3	25.6 ± 7.6	1
CRC Dukes B	7	3/4	65.3 ± 13.6	6
CRC Dukes C-D	8	4/4	67.5 ± 11.8	4
Normal	8	4/4	50.6 ± 5.97	5

### 2.2.2. Statistical analysis

#### *Pre-processing and quality control*

Quality control analyses were performed according to the suggestions of The Tumor Analysis Best Practices Working Group [37]. Scanned images were inspected for artifacts, percentage of present calls (> 25%) and control of the RNA degradation were evaluated. Based on the evaluation criteria all biopsy measurements fulfilled the minimal quality requirements. According to the above recommendations we have applied two different normalization methods: MAS 5.0, and RMA [18]. MAS5.0 applies normalization on an individual chip; it has excellent specificity and good sensitivity. RMA applies cross-project normalization; it has good specificity and excellent sensitivity [37]. Further data analysis and -interpretation have been carried out with both of these pre-processing methods in order to yield the best comparison and normalization properties across all measurements.

#### *Feature selection and cluster analysis*

We have arranged the complete dataset consisting of 52 expression measurements into classes according to the histological properties of the samples. This selection procedure resulted in six new datasets (CRC/adenoma/IBD vs. normal, CRC DukesB vs. CRC DukesC-D, non-dysplastic adenoma vs. dysplastic adenoma, UC vs. CD), which were treated as autonomous classification problems. In order to obtain characteristic signal profiles with high predictive power we have applied the "Prediction Analysis for Microarrays" (PAM) [35]. PAM uses soft thresholding to produce a shrunken centroid, which allows the selection of genes with high predictive potential. In our experimental setup the search for a minimum number of genes with maximum predictive accuracy is not promising as we could distinguish two different groups with a very short gene list. Therefore we decided to set the PAM threshold lower for the selection of the top genes and to pick the top 100 genes for each condition. Due to the nature of PAM, at lower threshold the resulting gene list will be longer, but all genes significant at a higher threshold

will be included in any selected set. Finally, the overlap of the two lists –based on two different normalizations– was taken for further analysis. The functional classification of the most differentially expressed genes were performed according to the analysis of RMA top 100 genes in each main disease groups compared to normal controls.

To visualize the discriminative patterns, hierarchical clustering was performed using the Genesis software [34]. Annotation was performed using the Affymetrix Netaffx analysis centre (<http://www.affymetrix.com/analysis/index.affx>). The combined datasets for further analysis are available in the Gene Expression Omnibus databank (<http://www.ncbi.nlm.nih.gov/geo/>), series accession number: GSE4183.

#### *Discriminant analysis*

Further data analysis was performed in the SAS v.8.2 (SAS Institute Inc. Cary, NC, USA) program package and the discriminant analysis in SPSS v.15.0 program (SPSS Inc., USA). We have performed a stepwise discriminant analysis among the groups by forward selection of quantitative variables. The set of variables that make up each class was assumed to be multivariate normal with a common covariance matrix. Finally we used the significant discrimination model (Wilks' Lambda was significant) and we have fixed the most important discriminatory genes. At the end of the analysis we have made the Leave-one-out classification table.

#### 2.2.3. Taqman RT-PCR

TaqMan real-time PCR was used to measure the expression of 52 selected genes using an Applied Biosystems Micro Fluidic Card System in 36 samples, where sufficient RNA could be extracted (Table 1). The measurements were performed using an ABI PRISM® 7900HT Sequence Detection System as described in the products User Guide (<http://www.appliedbiosystems.com>, CA, USA). For data analysis the SDS 2.2 software was used. The extracted delta Ct values (which represent the expres-

sion normalized to the ribosomal 18S expression) were grouped according to the histological groups. Then the Student's t-test was performed to compare the expression values between groups.

### 3. Results

#### 3.1. Identification of discriminatory genes among the main diagnostic groups

CRC cases are characterized by differentially expressed genes involved in cell proliferation (pleiotrophin, insulin-like growth factor binding protein 5, REG1A, WNT1 inducible signaling pathway protein 1), adhesion (MCAM, collagens, enactin, laminin gamma 1), and transport (such as aquaporin 8, lipocalin 2 and collagen 4 A1). Adenoma cases showed altered gene expression data in transport (like ABCA8, TRPM6), adhesion (such as CXCL12, CD44, ADAM-like decysin 1), metabolism (like carbonic anhydrase I) and proliferation (such as MET protooncogene) functional groups. IBD cases are featured by the gene expression changes of immune regulation (such as IFITM3, IFITM1 and proteasome subunit, beta type, 9), cell proliferation (REG1A, tryptophanyl-tRNA synthetase, interferon stimulated gene 20kDa) and metabolism (like chitinase 3-like 1, carbonic anhydrase I, zinc finger protein 91). The top 100 genes for each condition were picked and the overlaps of the two lists, based on two different normalizations were determined. Discriminatory genes between CRC/adenoma/IBD and normal biopsy samples are listed in Table 2, and considered as the disease type specific mRNA expression marker groups. The list of the top 100 genes for each analysis setting including complete annotation and the complete microarray dataset are shown as supplementary information in Supplemental Table 2. The average within-PAM cross-validation misclassification error was found to be 7.3%.

To visualize the expression changes, we have clustered the selected top genes of all biopsy samples to detect similarities across the sample groups (Fig. 1). The dendrogram of the 52 colonic cases shows the discrimination potential of the selected genes. The transcripts representing the same gene are found to fit into the same cluster.

#### 3.2. Discriminant analysis of the array results

We were able to classify the samples using 7 discriminatory genes (indoleamine-pyrrole 2,3 dioxygenase, ectodermal-neural cortex, TIMP3, fucosyltransferase 8, collectin sub-family member 12, carboxypeptidase D, and transglutaminase 2 (Fig. 2). 96.2% of original grouped cases and 94.3 % of cross-validated grouped cases were correctly classified (Table 3).

#### 3.3. Discrimination of colonic disease subtypes

The subclassifying genes within the main disease groups were also identified. The metabolic and transport processes (representing genes like DnaJ homolog subfamily C member 10, coatomer protein complex subunit beta) mainly differ between CRC subgroups. In advanced stages of CRC downregulation of apoptosis (such as TIA1 cytotoxic granule-associated RNA binding protein, forkhead box O3A), and immune response (like immunoglobulin heavy constant mu, 2'-5'-oligoadenylate synthetase 2) was observed, while carbohydrate, fatty acid metabolism (like glutamine-fructose-6-phosphate transaminase 1, GDP-mannose 4,6-dehydratase, sterol-C4-methyl oxidase-like, lanosterol synthase) and energy metabolism related genes (such as ATPase inhibitory factor 1) showed higher mRNA expression levels in parallel with CRC progression. In adenoma samples upregulation of proliferation (such as interferon gamma-inducible protein 16, aminopeptidase A, tumor protein D52-like 2) and DNA replication and transcription regulation (like IGF1, nuclear factor-like 3, zinc finger protein 452) and downregulation of immune and defense response (such as immunoglobulin heavy constant mu, T cell receptor gamma variable 9, interferon regulatory factor 4 and tryptase alpha) were found during the development of dysplastic alterations. CD cases are mainly featured by increased expression of carbohydrate metabolism genes (like galactosidase alpha, fructose-2,6-biphosphatase 4, maltase-glucoamylase), while certain cell proliferation (such as septin 10, platelet derived growth factor D, cyclin G2), apoptosis (such as BCL10, BIRC4, egl nine homolog 3), immune regulation (like decay accelerating factor, CD24, CEACAM1), transport (like dual oxidase 2, P450 (cytochrome) oxidoreductase and lipocalin 2), and ubiquitin-dependent protein catabolism (such as tripartite motif-containing 31, ubiquitin-conjugating enzyme E2, J1, and Ubiquitin specific protease 53) genes were found to be overexpressed in UC compared with CD cases. However, the function of many sub-

Table 2

Genes differentially expressed in CRC/adenoma/IBD compared to normal mucosa (overlap of genes identified using PAM after both RMA and MAS 5.0 normalizations)

	Probe Set ID	Gene Name	Gene Symbol	Gene Ontology Biological Process	Fold change
CRC vs normal	202112_at	von Willebrand factor	VWF	cell adhesion / platelet activation / response to wounding	3.61
	211980_at	collagen, type IV, alpha 1	COL4A1	phosphate transport	5.21
	212531_at	lipocalin 2 (oncogene 24p3)	LCN2	transport	7.97
	240157_at	—	—	—	-6.30
	220724_at	hypothetical protein FLJ21511	FLJ21511	—	-4.08
	220723_s_at	hypothetical protein FLJ21511	FLJ21511	—	-4.07
	203065_s_at	caveolin 1, caveolae protein, 22kDa	CAV1	—	2.70
	212768_s_at	olfactomedin 4	OLFM4	—	3.70
	223587_s_at	amionless homolog (mouse)	AMN	development	-3.91
	206784_at	aquaporin 8	AQP8	water transport	-5.02
	215894_at	prostaglandin D2 receptor (DP)	PTGDR	G-protein coupled receptor protein signaling pathway	-3.30
	214845_s_at	calumenin	CALU	—	3.23
	213119_at	solute carrier family 36 (proton/amino acid symporter), member 1	SLC36A1	amino acid transport	-2.27
	Adenoma vs normal	204719_at	ATP-binding cassette, sub-family A (ABC1), member 8	ABCA8	transport
230204_at		—	—	—	-4.05
203510_at		met proto-oncogene (hepatocyte growth factor receptor)	MET	cell proliferation/ cell surface receptor linked signal transduction / protein amino acid phosphorylation	3.91
240389_at		—	—	—	-13.50
1552296_at		vitelliform macular dystrophy 2-like 2	VMD2L2	ion transport	-11.34
224412_s_at		transient receptor potential cation channel, subfamily M, member 6	TRPM6	cation transport / protein amino acid phosphorylation	-11.59
209687_at		chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	CXCL12	G-protein coupled receptor protein signaling pathway / calcium ion homeostasis /cell adhesion / cell-cell signaling	-6.91
212014_x_at		CD44 antigen (homing function and Indian blood group system)	CD44	cell adhesion /cell-cell adhesion / cell-matrix adhesion	3.87
205523_at		hyaluronan and proteoglycan link protein 1	HAPLN1	cell adhesion	-5.04
206134_at		ADAM-like, decysin 1	ADAMDEC1	integrin-mediated signaling pathway/ negative regulation of cell adhesion / proteolysis and peptidolysis	-6.81
209835_x_at		CD44 antigen (homing function and Indian blood group system)	CD44	cell adhesion /cell-cell adhesion /cell-matrix adhesion	3.74
205950_s_at		carbonic anhydrase I	CA1	one-carbon compound metabolism	-9.77
228507_at		Phosphodiesterase 3A, cGMP-inhibited	PDE3A	lipid metabolism /signal transduction	-4.36
225275_at		EGF-like repeats and discoidin I-like domains 3	EDIL3	cell adhesion / development	-4.38
203000_at		stathmin-like 2	STMN2	intracellular signaling cascade /neuron differentiation	-5.17
236300_at		Phosphodiesterase 3A, cGMP-inhibited	PDE3A	lipid metabolism /signal transduction	-3.85
220724_at		hypothetical protein FLJ21511	FLJ21511	—	-5.26
207504_at		carbonic anhydrase VII	CA7	one-carbon compound metabolism	-8.08
204351_at		S100 calcium binding protein P	S100P	—	4.38
229839_at		hypothetical protein MGC45780	MGC45780	phosphate transport	-5.04
202242_at		transmembrane 4 superfamily member 2	TM4SF2	N-linked glycosylation	-3.74
235849_at		hypothetical protein MGC45780	MGC45780	phosphate transport	-4.59
218756_s_at		hypothetical protein MGC4172	MGC4172	metabolism	-3.65
215657_at		—	—	—	-7.77
215177_s_at		integrin, alpha 6	ITGA6	cell-matrix adhesion / cell-substrate junction assembly /integrin-mediated signaling pathway	2.96
204931_at		transcription factor 21	TCF21	mesoderm development /regulation of transcription, DNA-dependent	-3.20

Table 2, continued

	Probe Set ID	Gene Name	Gene Symbol	Gene Ontology Biological Process	Fold change
	207003_at	guanylate cyclase activator 2A (guanylin)	GUCA2A	signal transduction	-7.55
IBD vs normal	212203_x_at	interferon induced transmembrane protein 3 (1-8U)	IFITM3	immune response / response to biotic stimulus /	3.41
	204279_at	proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional protease 2)	PSMB9	immune response / proteolysis and peptidolysis / ubiquitin-dependent protein catabolism	3.77
	240389_at	—	—	—	-7.81
	201601_x_at	interferon induced transmembrane protein 1 (9-27)	IFITM1	cell surface receptor linked signal transduction/immune response /negative regulation of cell proliferation	3.32
	214022_s_at	interferon induced transmembrane protein 1 (9-27)	IFITM1	cell surface receptor linked signal transduction /immune response /negative regulation of cell proliferation	2.62
	201649_at	ubiquitin-conjugating enzyme E2L 6	UBE2L6	ubiquitin cycle	3.31
	206059_at	zinc finger protein 91 (HPF7, HTF10)	ZNF91	carbohydrate metabolism / regulation of transcription, DNA-dependent	-2.22
	224412_s_at	transient receptor potential cation channel, subfamily M, member 6	TRPM6	cation transport /protein amino acid phosphorylation	-7.06
	213369_at	—	—	—	-6.27
	212531_at	lipocalin 2 (oncogene 24p3)	LCN2	transport	10.96

type discriminatory genes has not been identified yet. Best differentiating markers between UC and CD were cyclin-G2, tripartite motif-containing-31, TNFR shedding aminopeptidase regulator-1 and AMICA.

The functional classification of most differentially expressed genes was performed according to the analysis of RMA top 100 genes in each disease type subgroups. The list of the top 100 genes for each analysis setting including complete annotation and the complete microarray dataset are shown as supplementary information (Supplemental Table 2). Hierarchical cluster diagrams of the subgroups, based on RMA top 100 differentially expressed genes can be seen on Fig. 3.

### 3.4. Gene ontology of selected features

The representative gene ontology categories are shown to Tables 2 and 3. We have also mapped the selected features to chromosomes (Supplemental Fig. 1).

### 3.5. Taqman validation

Selection criteria for genes were the different expression in microarray analysis and the availability of validated TaqMan probes. Ten “literature” genes were also selected which were described as CRC related genes. The complete results of the TaqMan measurements are presented on Supplemental Table 3. Forty six of the 52 measured genes correlated with the results obtained using Affymetrix microarrays at a significance of  $p < 0.05$ . The expression changes of the selected genes are

summarized in Table 4. The mRNA expression levels of selected discriminatory genes measured by Taqman RT-PCR are presented on Supplemental Fig. 2. Global clustering of all samples using the Taqman validated genes were also performed (Supplemental Fig. 3). Normal and UC cases belong to two distinct clusters, but clusters of CRC and adenoma cases are not clearly separated, demonstrating the expressional heterogeneity of CRC.

## 4. Discussion

Gene expression profiling of 52 colonic biopsy samples was done by whole genomic HGU133 Plus 2.0 microarrays in order to identify disease specific gene expression marker groups for objective classification. We aimed to develop diagnostic mRNA expression patterns for identification of adenoma and different staged CRC and of the minimal list of genes which is suitable for discrimination of different types of IBD. Examination of adenoma with/without dysplasia, CRC, IBD and normal samples in parallel can help to find condition specific gene expression alteration with a lower risk of unspecificity due to methodical reasons. Comparative microarray analysis of biopsies from all of these kinds of colonic diseases has not been reported so far in the scientific literature. Oligonucleotide whole genomic microarray analyses of biopsy samples were found to be highly standardized, reproducible and provided high quality array results regarding the array

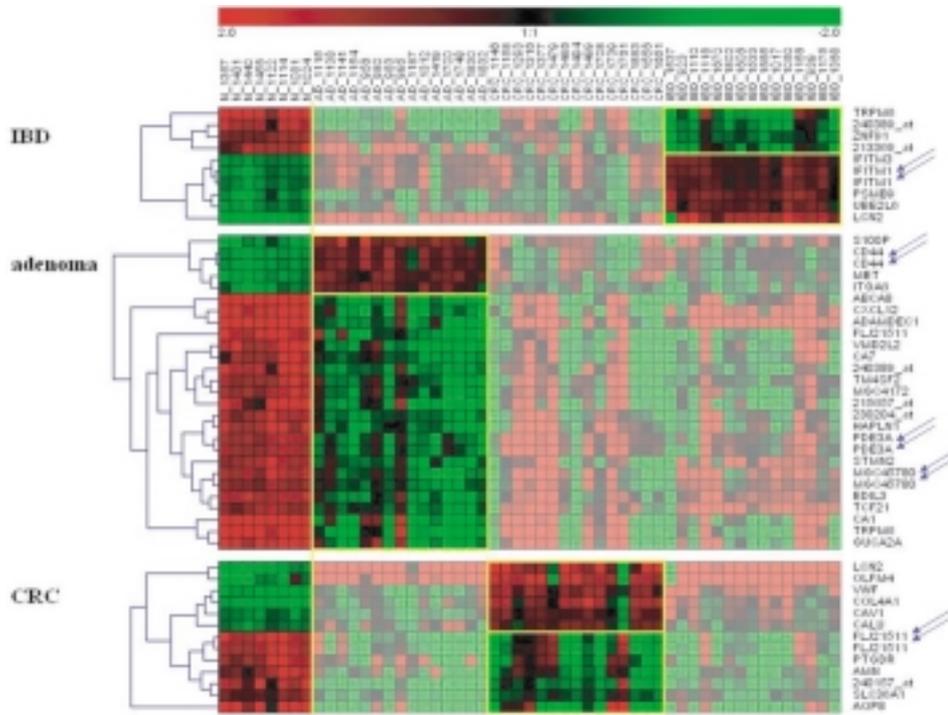


Fig. 1. Combined clustering dendrogram of the top dicriminative transcripts for IBD, adenoma and CRC. The disease samples were grouped, normalized and compared to normal controls. Samples, not included in the classification of selected disease are shaded. Repeated transcripts are marked with arrows.

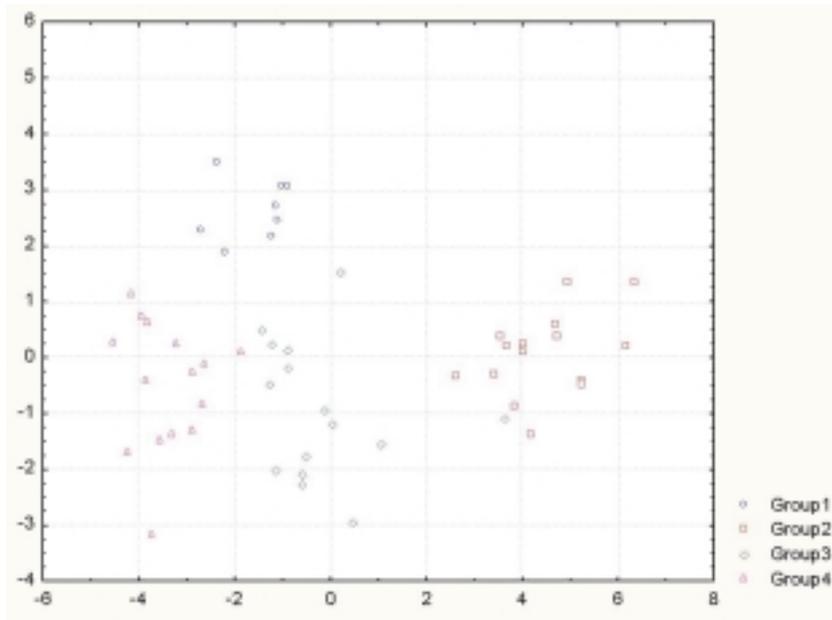


Fig. 2. Discriminant analysis of colonic biopsy specimens. Note the clear separation of the single classification groups based on the discriminatory genes detailed in the results section. Group 1 = normal, Group 2 = adenoma, Group 3 = colorectal cancer, Group 4 = inflammatory bowel disease.

Table 3  
Classification results<sup>b,c</sup>

	Group	Predicted Group Membership				Total	
		1	2	3	4		
Original	Count	1	8	0	0	0	8
		2	0	15	0	0	15
		3	0	1	14	0	15
		4	0	0	1	14	15
	%	1	100.0	0	0	0	100.0
		2	0	100.0	0	0	100.0
		3	0	6.7	93.3	0	100.0
		4	0	0	6.7	93.3	100.0
Cross-validated <sup>a</sup>	Count	1	8	0	0	0	8
		2	0	15	0	0	15
		3	1	1	13	0	15
		4	0	0	1	14	15
	%	1	100.0	0	0	0	100.0
		2	0	100.0	0	0	100.0
		3	6.7	6.7	86.7	0	100.0
		4	0	0	6.7	93.3	100.0

<sup>a</sup>Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

<sup>b</sup>96.2% of original grouped cases correctly classified.

<sup>c</sup>94.3% of cross-validated grouped cases correctly classified.

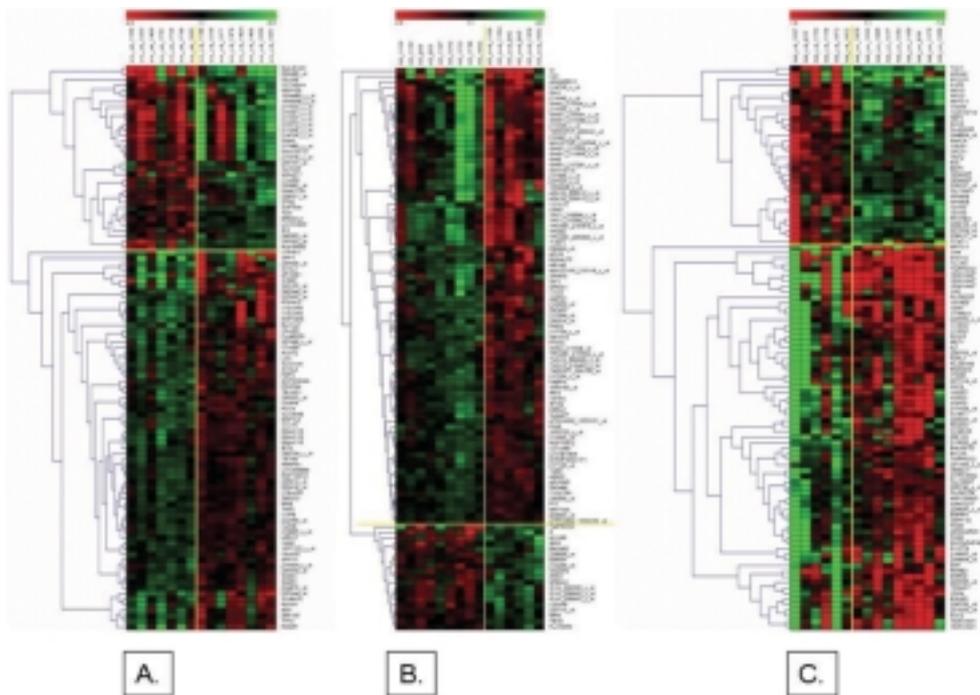


Fig. 3. Discriminating disease subtypes. Hierarchical clustering of genes associated with disease subtype-differentiation using the top 100 differentially expressed genes obtained after RMA normalization. A. CRC Dukes B vs. CRC Dukes C-D. B. Adenoma without dysplasia vs adenoma with dysplasia. C. Ulcerative colitis vs Crohn's disease.

Table 4  
Taqman measurement of 52 genes

Taqman ID	Gene Symbol	Gene name	Affymetrix ID	Sample groups	p value	ddCt	Fold change in microarrays
Hs00153304_m1	CD44	CD44 antigen	212014_x_at	AD vs normal	<b>1.82E-07</b>	1.903.87	
Hs00157859_m1	GUCA2A	guanylate cyclase activator 2A	207003_at	AD vs normal	<b>0.000411</b>	-4.34	-7.55
				CRC vs normal	<b>0.00871</b>	-3.17	-2.33
Hs00171022_m1	CXCL12	chemokine (C-X-C motif) ligand 12	209687_at	AD vs normal	<b>0.00305</b>	-2.04	-6.91
				CRC vs normal	<b>0.00735</b>	-1.95	-2.32
Hs00179845_m1	MET	met proto-oncogene	203510_at	AD vs normal	<b>1.41E-06</b>	2.17	3.91
Hs00200350_m1	ABCA8	ATP-binding cassette, sub-family A (ABC1), member 8	204719_at	AD vs normal	<b>0.000610</b>	-3.35	-12.78
				UC vs normal	<b>8.07E-05</b>	-2.70	-2.27
Hs00205545_m1	ADAMDEC1	ADAM-like, decysin 1	206134_at	AD vs normal	<b>1.16E-05</b>	-3.69	-6.81
				CRC vs normal	<b>9.18E-05</b>	-2.74	-2.26
				UC vs normal	<b>0.00439</b>	-1.21	-1.27
Hs00214306_m1	TRPM6	transient receptor potential cation channel, subfamily M, member 6	224412_s_at	AD vs normal	<b>5.79E-05</b>	-4.73	-11.59
				CRC vs normal	<b>0.00827</b>	-3.09	-2.73
				UC vs normal	<b>0.000385</b>	-4.63	-3.48
Hs00153408_m1	MYC	v-myc myelocytomatosis viral onco-gene homolog (avian)	202431_s_at	AD vs normal	<b>5.99E-06</b>	2.35	2.03
Hs00163869_m1	CA2	carbonic anhydrase II	209301_at	AD vs normal	<b>0.000494</b>	-3.75	-3.24
				CRC vs normal	<b>0.00193</b>	-3.17	-2.14
Hs00171558_m1	TIMP1	tissue inhibitor of metalloproteinase 1	201666_at	AD vs normal	<b>3.90E-07</b>	2.58	1.95
				CRC vs normal	<b>0.00153</b>	2.74	3.40
				UC vs normal	<b>0.000219</b>	2.36	2.03
Hs00236937_m1	CXCL1	chemokine (C-X-C motif) ligand 1	204470_at	AD vs normal	<b>1.66E-05</b>	3.55	5.38
				CRC vs normal	<b>0.0114</b>	3.84	13.10
				UC vs normal	<b>1.11E-05</b>	4.04	4.78
Hs00236966_m1	CXCL2	chemokine (C-X-C motif) ligand 2	209774_x_at	AD vs normal	<b>1.24E-05</b>	3.98	5.49
				CRC vs normal	<b>0.00204</b>	3.70	9.20
				UC vs normal	<b>0.000592</b>	3.68	3.94
Hs00266139_m1	CA1	carbonic anhydrase I	205950_s_at	AD vs normal	<b>0.000930</b>	-6.13	-9.77
Hs00605175_m1	ADH1B	alcohol dehydrogenase IB (class I), beta polypeptide	209613_s_at	AD vs normal	<b>0.00382</b>	-3.49	-4.27
Hs00154124_m1	AQP8	aquaporin 8	206784_at	AD vs normal	<b>0.000378</b>	-6.10	-6.20
				CRC vs normal	<b>0.0315</b>	-5.33	-5.02
Hs00194353_m1	LCN2	lipocalin 2	212531_at	AD vs normal	<b>2.67E-07</b>	6.13	4.85
				CRC vs normal	<b>0.000509</b>	4.83	7.97
				UC vs normal	<b>2.15E-06</b>	5.06	3.62
Hs00197437_m1	OLFM4	olfactomedin 4	212768_s_at	AD vs normal	<b>2.57E-07</b>	7.04	4.28
				CRC vs normal	<b>0.000179</b>	6.20	3.70
				UC vs normal	<b>4.99E-05</b>	5.68	
Hs00154230_m1	CALU	calumenin	214845_s_at	CRC vs normal	<b>0.0145</b>	1.60	3.23
Hs00169795_m1	VWF	von Willebrand factor	202112_at	CRC vs normal	0.551		3.61
				UC vs normal	<b>0.000112</b>	2.44	2.75
Hs00229558_m1	AMN	amniotomless homolog (mouse)	223587_s_at	CRC vs normal	<b>0.0499</b>	-2.32	-3.91
Hs00235003_m1	PTGDR	prostaglandin D2 receptor	215894_at	CRC vs normal	0.997	-3	.30
Hs00266237_m1	COL4A1	collagen, type IV, alpha 1	211980_at	CRC vs normal	<b>0.0283</b>	3.38	5.21
Hs00156076_m1	BGN	biglycan	213905_x_at	CRC vs normal	0.120		2.24
Hs00169777_m1	PECAM1	platelet/endothelial cell adhesion molecule	208983_s_at	CRC vs normal	0.764		1.59
Hs00174103_m1	IL8	interleukin 8	202859_x_at	CRC vs normal	<b>0.0283</b>	7.21	20.20
				UC vs normal	<b>6.80E-06</b>	5.77	4.13
Hs00204187_m1	DUOX2	dual oxidase 2	219727_at	AD vs normal	<b>5.77E-06</b>	5.25	6.00
				CRC vs normal	<b>0.00363</b>	4.91	9.70
				UC vs normal	<b>7.84E-05</b>	6.35	6.43
Hs00160066_m1	PI3	protease inhibitor 3, skin-derived (SKALP)	203691_at	UC vs normal	<b>0.000257</b>	4.26	3.40
Hs00195812_m1	LIPG	lipase, endothelial	219181_at	UC vs normal	<b>0.000588</b>	1.35	1.82
Hs00197374_m1	UBD	ubiquitin D	205890_s_at	UC vs normal	<b>0.000261</b>	3.20	2.97

Table 4, continued

Taqman ID	Gene Symbol	Gene name	Affymetrix ID	Sample groups	p value	ddCt	Fold change in microarrays
Hs00829485_sH	IFITM2	interferon induced transmembrane protein 2 (1-8D)	201315_x.at	UC vs normal	<b>0.00287</b>	1.66	1.65
Hs00171061_m1	CXCL3	chemokine (C-X-C motif) ligand 3	207850_at	UC vs normal	<b>7.48E-05</b>	3.58	3.41
Hs00194145_m1	HMGCS2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	204607_at	UC vs normal	<b>0.0104</b>	-3.15	-2.14
Hs00234579_m1	MMP9	matrix metalloproteinase 9	203936_s.at	UC vs normal	<b>0.00724</b>	1.85	2.07
Hs00277299_m1	IL1RN	interleukin 1 receptor antagonist	212657_s.at	UC vs normal	<b>1.10E-05</b>	5.30	3.03
Hs00165949_m1	TIMP3	tissue inhibitor of metalloproteinase 3	201150_s.at	CRC vs normal	0.274		1.50
				CRC vs AD	<b>0.000150</b>	1.14	1.49
Hs00234160_m1	SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	200665_s.at	CRC vs normal	0.617		2.90
				CRC vs AD	<b>0.00176</b>	1.36	1.51
Hs00162613_m1	TCF4	transcription factor 4	212386_at	CRC vs AD	<b>0.000982</b>	0.86	1.42
Hs00181211_m1	IGFBP3	insulin-like growth factor binding protein 3	210095_s.at	CRC vs AD	<b>0.000216</b>	1.56	1.57
Hs00189021_m1	CALD1	caldesmon 1	212077_at	CRC vs AD	<b>0.000188</b>	1.24	1.53
Hs00190740_m1	SPARCL1	SPARC-like 1 (mast9, hevyn)	200795_at	CRC vs AD	<b>0.000165</b>	1.29	1.62
Hs00249930_s1	RBMS1	RNA binding motif, single stranded interacting protein 1	215127_s.at	CRC vs AD	<b>0.00566</b>		1.52
Hs00255962_m1	TNS	tensin	221748_s.at	CRC vs AD	<b>0.000278</b>	1.15	1.63
Genes from the literature							
Hs00153350_m1	BCL2	B-cell CLL/lymphoma 2	203684_s.at	CRC vs normal	0.33308		
Hs00153353_m1	BIRC5	baculoviral IAP repeat-containing 5 (survivin)	202095_s.at	CRC vs normal	<b>0.02067</b>	1.51	1.36
Hs00170248_m1	THBS2	thrombospondin 2	203083_at	CRC vs normal	0.05582		
Hs00171257_m1	TGFB1	transforming growth factor, beta 1	203085_s.at	CRC vs normal	0.47079		
Hs00173626_m1	VEGF	vascular endothelial growth factor	210513_s.at	CRC vs normal	<b>0.04743</b>	1.69	1.29
Hs00181385_m1	IGFR1	insulin-like growth factor receptor 1	203627_at	CRC vs normal	0.31769		
Hs00193306_m1	EGFR	epidermal growth factor receptor	156548_s.at	CRC vs normal	0.53486		
Hs00361600_m1	GUCA1B	guanylate cyclase activator 1B (retina)	207003_at	CRC vs normal	0.73175		
Hs00426322_m1	ACHY	S-adenosylhomocysteine hydrolase	200903_s.at	CRC vs normal	<b>0.00581</b>	1.32	1.34
Hs00162669_m1	TERT	telomerase reverse transcriptase	207199_at	CRC vs normal	0.80232		

P value represents the correlation to the microarray data. ddCt represents the expression difference normalized to the ribosomal 18S expression. The significant differential expression ( $p < 0.05$ ) is marked with bold.

sensitivity, present percentage and GAPDH 3'/5' ratio. This type of analysis results in discriminative signatures, and gives an insight into the pathophysiological background of colonic diseases, and additionally, provides a data warehouse which can be further mined for in-depth pathway analyses. As recently described, joint analysis is more efficient than replication based analysis for two-stage genome-wide association studies [33]. Therefore we used a one-stage genome wide analysis to identify relevant gene expression signatures. For a classification problem comparable to our study a previous estimation suggested a required sample size of 51 subjects to detect a 2-fold change of expression level at  $\alpha = 0.001$  at the 90th percentile [41].

The main disease groups were individually compared to healthy controls. CRC samples were unequivocally distinguished according to the expression level of 13 genes. Six of them were validated by Taqman RT-PCR. Among the discriminatory genes lipocalin 2 (LCN2), collagen 4 alpha 1 (COL4A1) and aquaporin

8 (AQP8) were mentioned earlier as CRC-associated genes. LCN2 transport molecule acts as chemotactic agent and also regulates the matrix metalloproteinase-9 activity. AQP8 water channel protein is a marker for non-proliferative colonic epithelial cells, but it is not expressed by adenoma and CRC in protein level. In correlation with the findings of Fischer et al., lower AQP8 mRNA level was found in CRC and adenoma samples in our study also [13].

Adenoma cases were characterized and distinguished according to the expression changes of 27 overlapping genes. Seven of them (overexpressed CD44 and MET, and underexpressed GUCA2A, CXCL12, TRPM6, ABCA8, ADAMDEC1) were confirmed by Taqman RT-PCR. CD44 cell surface glycoprotein antigen is a receptor for hyaluronic acid, which can also bind osteopontin, EGFR, matrix metalloproteases and IGFBP3. The expression changes of CD44 can affect several different cellular pathways including EGFR-related proliferation and tumorigenesis, tumor tissue

remodeling and immune processes. Hepatocyte growth factor receptor (MET), which was found to be overexpressed both in colon adenoma and CRC, may play an important role in colorectal tumorigenesis. Similarly to the results of Trovato et al., in our study elevated c-met mRNA level was observed both in adenoma and CRC biopsies, but CRC samples showed lower c-met expression than adenomas. Reduced expression of c-met can be associated to the progression of adenoma into carcinoma [36]. GUCA2A (guanylin) plays role in the regulation of ion transport in the colon. Expression of guanylin is downregulated in human intestinal adenomas, moreover, recent results suggest that loss of guanylin activity leads to or is a result of adenocarcinoma [8]. The chemokine ligand 12 (CXCL12) which was found underexpressed both in different carcinomas earlier [32] and adenoma samples in our study, regulates cAMP production and ion transport in intestinal epithelial cells [12]. These data can support that alterations in ion transport of the colon are involved in colorectal carcinogenesis. The exact cellular function and role in adenoma and tumor development of TRPM6, ABCA8 and ADAMDEC1 gene products have not yet been determined.

Several genes were found to show elevated mRNA level according to the adenoma-CRC sequence. TCF4 is a transcriptional factor involved in Wnt-signalling pathway which is altered in over 90% of CRCs. TCF4 participates in transcriptional regulation of genes associated with colon carcinogenesis including c-myc, cyclin D1, TCF1, PPAR $\delta$ , MMP7 and MDR1. Tensin 1 (TNS1) which was also overexpressed in CRC compared to adenoma samples can also induce JNK and p38 activation leading to increased cell survival. RBMS1 is another gene which was detected as being upregulated in CRC compared to adenoma, it is also involved in the malignant transformation process. RBMS1 is a modulator of c-myc, deregulates cell cycle controls and leads cells towards transforming pathways [28]. SPARC (osteonectin) was detected as overexpressed gene in CRC in several microarray-based studies [10, 29,42]. It is thought to play an important role in tissue remodeling, angiogenesis, and tumorigenesis. Controversial and conflicting data were published about the expression and function of insulin-like growth factor binding protein 3 and SPARCL1 in different types of cancers including CRC [19,20].

Ten discriminatory transcripts distinguish between

IBD samples and normal tissue. Overexpression of different interferon-induced genes is highly represented among the discriminatory genes. Interferon induced transmembrane protein 3 (IFITM3) was strongly expressed in severely inflamed colonic mucosa of UC both in our microarray analysis and other studies [17]. Moreover, IFITM3 showed high mRNA level in sporadic cancers, and UC-associated cancers, therefore it can be a marker for identification of high cancer-risk group within the UC. PSMB9 and UBE2L6 interferon-induced discriminatory genes are in connection with the enhanced antigen processing and presentation. LCN2 which has been mentioned above as an upregulated CRC-associated gene is also overexpressed in colonocytes and neutrophils in inflamed lesions of UC. Similarly to our findings, highly increased LCN2 mRNA levels were measured in UC samples in other microarray studies [11,24]. Alteration of epithelial magnesium absorption was also observed in our IBD samples, as the TRPM6 (transient receptor potential cation channel, subfamily member 8) showed lower mRNA level. CD cases are mainly featured by increased expression of carbohydrate metabolism genes, while certain cell proliferation, apoptosis, immune regulation, transport, and ubiquitin-dependent protein catabolism genes were found to be overexpressed in UC compared with CD cases. However, the function of many discriminatory genes has not been identified yet. Significant overexpression of cancer-related genes (CEACAM1, -7, CD24, PDGFD) in UC is potentially important, considering reports of increased risk of developing CRC in this disease [6,9].

For validation of the marker properties of a given gene, we should use homogeneous high case-number sample groups for microarray analysis, and further experiments, but at least RT-PCR confirmation, are needed. We focused on individual markers and independent validation of markers. Ninety-four percent of 52 selected genes which were found to be over- or underexpressed was confirmed by Taqman RT-PCR.

In conclusion, in our study we were able to distinguish not just between normal, adenoma, CRC and IBD samples, but also among the different stages of CRC using only easily-taken biopsy specimens. With a large number of samples one can establish principal gene lists that characterize distinct conditions, and if miniarrays will be commercially available, the daily routine of diagnosis may be quicker and easier.

## Supplemental

Supplement Table 1  
Patients characteristics

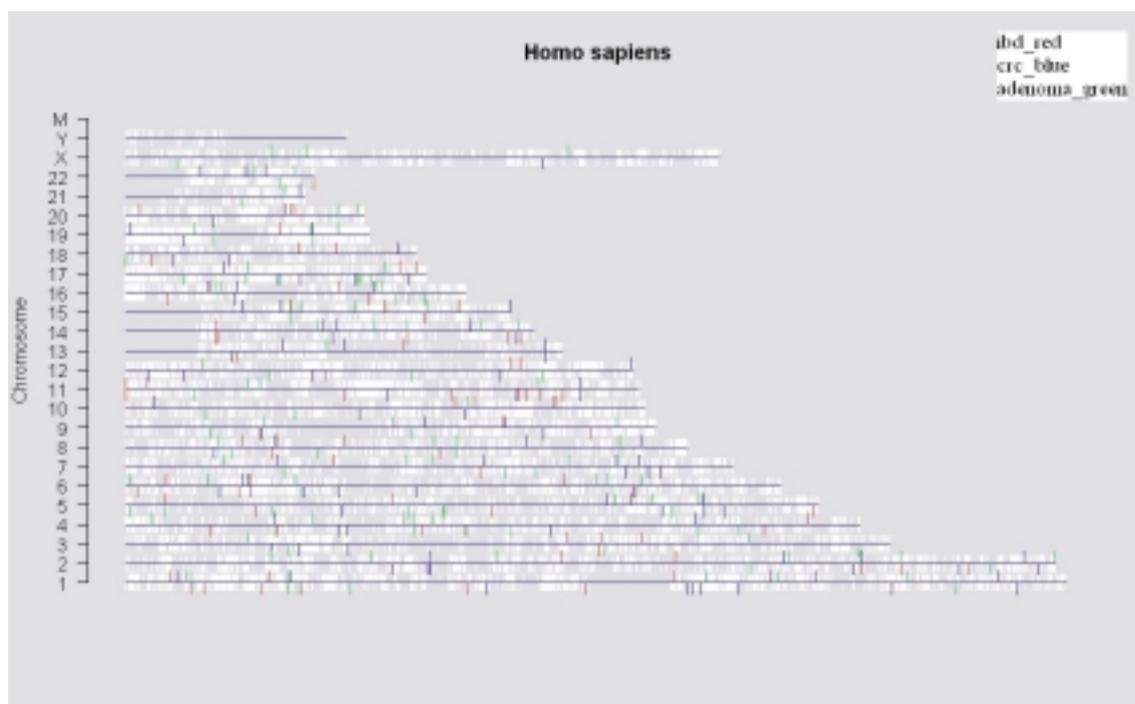
Sample	Affy ID	Sex	Age	Localization	Histology	TNM	Grade	Dukes	Dysplasia	Taqman analysis
<i>Adenoma</i>										
956*	956 U133 Plus 2.0 .CEL	F	84	flexure hepatica	adenoma villous				severe	
983*	983 U133 Plus 2.0 .CEL	F	64	rectum	adenoma tubulovillous				severe	Yes (3.)
1115*	1115 U133 Plus 2.0 .CEL	F	75	coecum	adenoma tubulovillous				severe	
1141	1141 U133 Plus 2.0 .CEL	M	63	rectum	adenoma tubulovillous				moderate	Yes (10.)
1187*	1187 U133 Plus 2.0 .CEL	F	80	coecum	adenoma tubulovillous				moderate	Yes (13.)
1312*	1312 U133 Plus 2.0 .CEL	M	93	rectum	adenoma villous				severe	Yes (15.)
1700*	1700 U133 Plus 2.0 .CEL	F	69	coecum	adenoma tubulovillous				severe	
1748*	1748 U133 Plus 2.0 .CEL	F	78	colon desc	adenoma villous				severe	Yes (32.)
1832*	1832 U133 Plus 2.0 .CEL	M	56	sigma-colon desc	adenoma villous				moderate	Yes (34.)
980	980 U133 Plus 2.0 .CEL	F	83	coecum	adenoma tubulovillous				no	Yes (2.)
995*	995 U133 Plus 2.0 .CEL	M	58	coecum, coecum, rectum	adenoma tubulovillous				no	Yes (4.)
1138*	1138 U133 Plus 2.0 .CEL	M	70	rectum	adenoma tubulovillous				no	Yes (9.)
1154*	1154 U133 Plus 2.0 .CEL	F	73	coecum	adenoma tubulovillous				no	Yes (11.)
1419*	1419 U133 Plus 2.0 .CEL	F	37	rectum	adenoma villous				no	Yes (19.)
1830*	1830 U133 Plus 2.0 .CEL	M	70	sigma	adenoma tubulovillous				no	Yes (33.)
<i>CRC</i>										
1158*	1158 U133 Plus 2.0 .CEL	M	56	rectum	adenocarcinoma	T3N0M0	G2	B2		Yes (12.)
1293*	1293 U133 Plus 2.0 .CEL	F	88	ascendent colon	adenocarcinoma	T3N0M0	G3	B2		Yes (14.)
1486*	1486 U133 Plus 2.0 .CEL	F	51	hepatic flexure	adenocarcinoma	T2N0M0	G1	B1		Yes (23.)
1708*	1708 U133 Plus 2.0 .CEL	F	72	sigma	adenocarcinoma	T3N0M0	G2	B2		Yes (30.)
1739*	1739 U133 Plus 2.0 .CEL	F	76	colon desc	adenocarcinoma	T4N0M0	G1	B3		Yes (31.)
1761	1761 U133 Plus 2.0 .CEL	M	58	rectum	adenocarcinoma	T2N0M0	G2	B1		
1883*	1883 U133 Plus 2.0 .CEL	M	56	coecum tumor	adenocarcinoma	T3N0M0	G2	B2		Yes (35.)
1146*	1146 U133 Plus 2.0 .CEL	M	46	descendent colon	adenocarcinoma	T2N1M0	G2	C1		
1316*	1316 U133 Plus 2.0 .CEL	M	85	lienic flexure	adenocarcinoma	T3N1M1	G2	D		Yes (16.)
1377*	1377 U133 Plus 2.0 .CEL	F	69	sigma	adenocarcinoma	T4N1M0	G1-2	C3		
1479*	1479 U133 Plus 2.0 .CEL	F	77	sigma	adenocarcinoma	T4N0M1	G1	D		
1494*	1494 U133 Plus 2.0 .CEL	M	73	rectum	adenocarcinoma	T3N1M1	G1	D		Yes (24.)
1499*	1499 U133 Plus 2.0 .CEL	M	65	rectum	adenocarcinoma	T3N0M1	G1	D		
1556*	1556 U133 Plus 2.0 .CEL	F	66	coecum	adenocarcinoma	T3N1M1	G2	D		Yes (26.)
1651	1651 U133 Plus 2.0 .CEL	F	59	rectum	adenocarcinoma	T2N0M1	G1	D		Yes (28.)
<i>IBD</i>										
939	939 U133 Plus 2.0 .CEL	F	34	sigma,rectum	severe active ulcerative colitis				no	Yes (1.)
1017	1017 U133 Plus 2.0 .CEL	F	39	total colon	severe active ulcerative pancolitis				no	Yes (5.)
1060	1060 U133 Plus 2.0 .CEL	F	73	total colon	severe active ulcerative colitis				no	Yes (7.)
1156	1156 U133 Plus 2.0 .CEL	M	45	sigma,rectum	severe active ulcerative colitis				no	
1175	1175 U133 Plus 2.0 .CEL	M	43	total colon	severe active ulcerative colitis				no	
1368	1368 U133 Plus 2.0 .CEL	F	24	total colon	severe active ulcerative colitis				mild	Yes (18.)
1533	1533 U133 Plus 2.0 .CEL	F	35	rectum,sigma	severe active ulcerative colitis				no	Yes (25.)
1606	1606 U133 Plus 2.0 .CEL	F	35	total colon	severe active ulcerative pancolitis				no	Yes (27.)
1665	1665 U133 Plus 2.0 .CEL	M	66	total colon, mainly sigma,rectum	severe active ulcerative colitis				mild-moderate	Yes (29.)

Supplement Table 1, continued

Sample	Affy ID	Sex	Age	Localization	Histology	TNM	Grade	Dukes	Dysplasia	Taqman analysis
923	923 U133 Plus 2.0 .CEL	F	37	sigma,rectum	severe active Crohn's disease				no	
1110	1110 U133 Plus 2.0 .CEL	F	16	total colon	severe active Crohn's disease				mild	Yes (8.)
1118	1118 U133 Plus 2.0 .CEL	M	26	right side	severe active Crohn's disease				no	
1670	1670 U133 Plus 2.0 .CEL	M	23	total colon	severe active Crohn's disease				no	
1802	1802 U133 Plus 2.0 .CEL	F	26	total colon	severe active Crohn's disease				no	
<i>Normal</i>										
1024	1024 U133 Plus 2.0 .CEL	F	47		normal					Yes (6.)
1081	1081 U133 Plus 2.0 .CEL	F	57		normal					
1114	1114 U133 Plus 2.0 .CEL	F	60		normal					
1122	1122 U133 Plus 2.0 .CEL	M	45		normal					
1357	1357 U133 Plus 2.0 .CEL	M	47		normal					Yes (17.)
1431	1431 U133 Plus 2.0 .CEL	M	50		normal					Yes (20.)
1440	1440 U133 Plus 2.0 .CEL	F	44		normal					Yes (21.)
1456	1456 U133 Plus 2.0 .CEL	M	55		normal					Yes (22.)

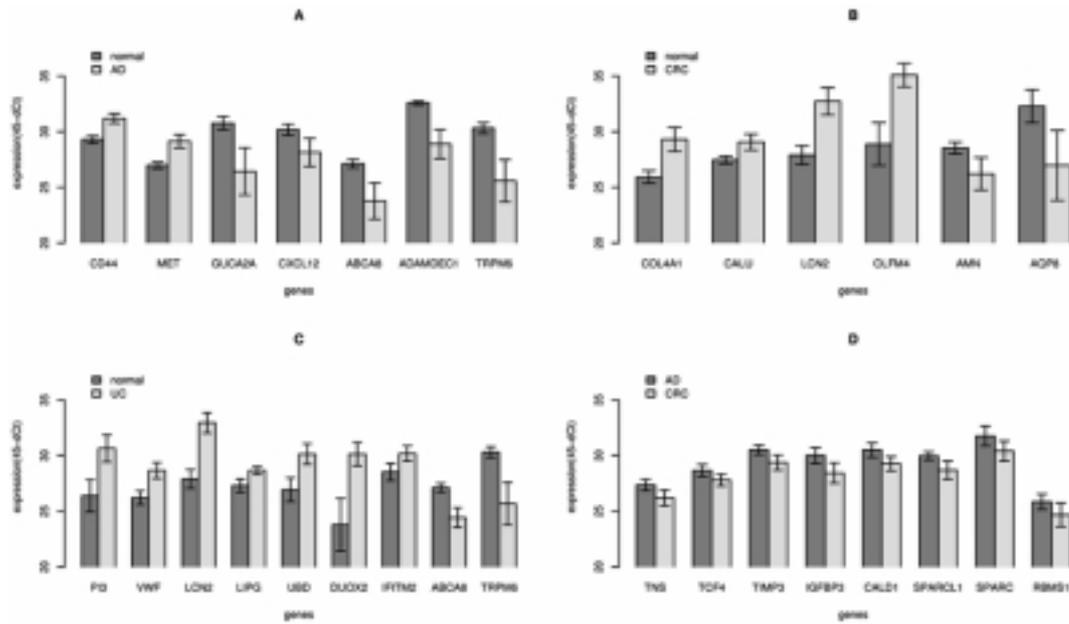
Microsatellite instability analysis was done from samples marked with \*.

GEO Accession numbers: "normal: GSM95473 – GSM95480", "adenoma: GSM95481 – GSM95495", "CRC: GSM95496 – GSM95510", "IBD: GSM95511 – GSM95525".

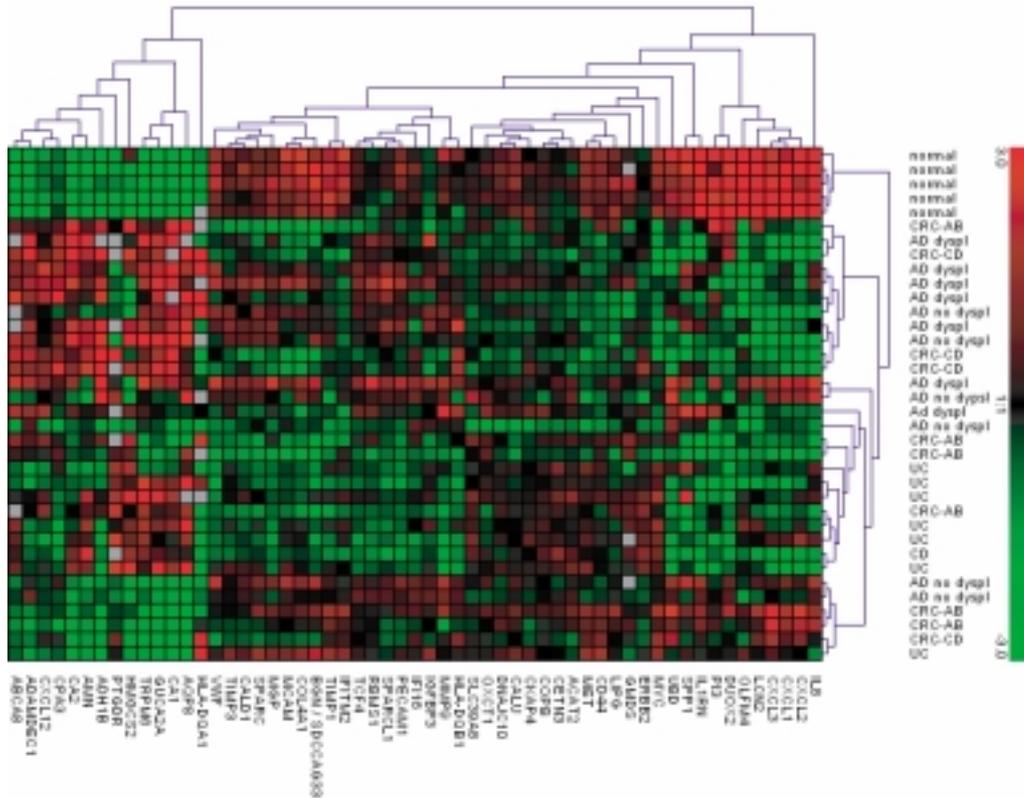


Supplement Figure 1. Chromosomal localization of discriminatory genes.

The Supplemental Table 2 and the Supplemental Table 3 are available on the <http://3dhitech.com/en/article/DMA-511> website.



Supplement Figure 2. mRNA expression levels of selected discriminatory genes measured by Taqman RT-PCR. dCt is the expression value normalized to the ribosomal 18S protein.



Supplement Figure 3. Global clustering of all samples measured by Taqman RT-PCR according to the Taqman validated genes. Probably due to the heterogeneity of CRC samples they do not cluster together during global clustering using Taqman-generated results.

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