

Peripheral blood based discrimination of ulcerative colitis and Crohn's disease from non-IBD colitis by genome-wide gene expression profiling

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Abstract. A molecular diagnostic assay using easily accessible peripheral blood would greatly assist in the screening and diagnosis of ulcerative colitis (UC) and Crohn's disease (CD). Transcriptional profiles in blood/biopsy samples from 12 UC (6/12), 9 CD (5/9), 6 non-inflammatory bowel disease (non-IBD) colitis (6/0), and 11 healthy (11/11) patients were assessed by Affymetrix HGU133Plus2.0 microarrays. Prediction analysis of microarrays, discriminant and ROC analyses were performed, the results were validated by RT-PCR and immunohistochemistry using also an independent set of samples (15 blood samples, 45 biopsies). A set of 13 transcripts was differentially expressed in IBD, non-IBD controls and healthy blood samples (100% specificity and sensitivity). Validated difference was found in 16 transcripts between UC, non-IBD and normal blood, and 4 transcripts between CD, non-IBD and normal samples. UC and CD blood cases could be also distinguished by 5 genes with 100% specificity and sensitivity. Some disease associated alterations in blood transcripts were also detected in colonic tissue. IBD subtypes may be discriminated from non-IBD (diverticulitis, infective and ischemic colitis) *in vitro* from peripheral blood by screening for differential gene expression revealed in this study. Transcriptional profile alterations in peripheral blood can be located in diseased colon.

Keywords: IBD, blood, gene expression profile, screening, tissue microarray

1. Introduction

The concept that ulcerative colitis (UC) and Crohn's disease (CD) are two distinct forms of inflammatory bowel diseases (IBD) has been challenged recently. Instead, they are considered as a spectrum from mildly inflamed colon to severely active bowel inflammation with or without extraintestinal manifestations and different clinical behavior.

Reliable diagnosis of IBD still requires biopsy samples. However, a screening method using samples of minimally invasive interventions such as peripheral blood for monitoring disease activity and the efficacy of therapy would be of great benefit. Conventional laboratory parameters used in daily clinical routine such as erythrocyte sedimentation rate, complete and differential blood count or CRP may have some diagnostic and/or prognostic relevance, but these are not specific for IBD or predictive for a therapeutic response. In addition, proposals for molecular and genetic classification of IBD has been made recently, which also requires more specific information [1–3]. Markers from the peripheral blood, such as anti-neutrophil cytoplasmic

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antibodies (ANCA) and anti-Saccharomyces cerevisiae antibodies (ASCA) [4]; and genetic polymorphism, most notably that of the NOD-like receptor family (i.e.: caspase activation recruitment domain 15 (CARD15) gene at chromosome 16), COX-2, glucocorticoid receptor, multidrug-resistance protein 4, or Toll-like receptors has been intensively investigated [5–12]. Moreover, subtypes of IBD cannot always be determined unambiguously even with conventional histopathology.

Clinical differentiation between UC and CD has long been a problem due to a substantial overlap not only in disease pathophysiology but also in the clinical course. Except some difference in their efficacy, most medical treatments used for colonic inflammation in IBD are applicable to both CD and UC [13]. A considerable percentage of patients appear to change clinical presentation during the course of the disease and are re-diagnosed with one of the other IBD subtypes. Based on the differences in natural history of IBD including the rate of surgery [14], or differences in post surgical outcomes, such as following ileal pouch-anal anastomosis [15], a molecular diagnostic assay using easily accessible samples of peripheral blood would greatly assist in the diagnostic screening and may be in the determination of IBD subtypes.

Since immune cells, e.g. peripheral blood leukocytes (PBL) recycle between the circulation and the epithelial and subepithelial layers of the human colon [16–19], tissue-specific biological information may be gained from the molecular screening of the peripheral blood. High-density oligonucleotide microarrays, used for testing gene expression profile changes between normal and diseased colonic tissues, may also be exploited for tracing if these changes occur in the peripheral blood [20,21]. However, not all PBL have clear relationship with the affected bowel tissue. Several oligonucleotide microarray [22–30] or subtractive suppression hybridization [31,32] studies using mRNA from gut biopsies identified genes involved in IBD and showed that GI tissue transcriptomes obtained from UC and CD patients were quite distinct.

To date, only a few studies were performed to classify UC and CD using PBL gene expression profiles [33, 34] or examine the leukocytapheresis-induced changes in PBLs gene expression patterns [35]. These studies, however, focused on the basic gene expression alterations between diseased and normal tissues using either 2400 gene cDNA microarray [34], 16600 gene TaKaRa IntelliGene HS human expression chip [35] and Affymetrix HG-U133A human GeneChip array with 22000 transcripts [33].

The aim of this study was to test if IBD subtype-specific gene expression profile alterations revealed with Affymetrix whole genom arrays, which can be identified both in peripheral blood and tissues. If established, differentially expressed genes of IBD could then be easily monitored from blood samples by using qRT-PCR both for convenient diagnostic screening and patient follow up.

2. Methods

2.1. Patients/Subjects and samples

After informed consent, pre-colonoscopy blood samples and colonic biopsy samples were taken from patients complaining of abdominal pain or cramps, frequent stool, bloody diarrhea or fever. 9 ml peripheral blood were taken and collected into Paxgene Blood RNA Tubes (Qiagen), which were stored at -20°C . Biopsy samples were stored in RNALater Reagent (Qiagen Inc, Germantown, US) at -80°C . Altogether 28 peripheral blood samples and 32 tissue samples of 12 active UC (5 male/ 7 female, average age 29.3 years), 9 active CD (4 male/ 5 female, average age 26.5 years), 4 diverticulitis (2 male/2 female, average age 57.4 years), 1 infective colitis (1 male, 28 years), 1 ischemic colitis (1 male, 62 years) and 11 healthy control patients (6 male/ 5 female, average age 36.4 years) were investigated. The diagnosis of active UC or CD was established on the basis of clinical symptoms (≥ 6 bloody stools per day, presence/absence of fever, tachycardia, abdominal pain), conventional laboratory findings (elevated erythrocyte sedimentation rate and CRP, thrombocytosis, mild-moderate anemia, presence/absence of hypokalemia and/or hypomagnesemia, negative stool culture) and this first colonoscopy, therefore none of the patients had been given corticosteroids, antibiotics or immunosuppressive treatment prior to samples being taken.

Before treatment, blood samples of 4 patients with diverticulitis, 1–1 patients of infective- (pseudomembranous) and ischemic colitis were collected as non-IBD colitis controls.

Histologically normal biopsy samples from the endoscopically healthy colon and pre-colonoscopy peripheral blood samples of healthy control patients undergoing colonoscopy because of a positive fecal occult blood test (FOBT) were used as healthy controls. The FOBT had been performed for screening purposes. In the healthy control group, there was no

pathological colon alteration apart from internal hemorrhoids. The percentage of left-/right colon sided biopsies was 81.25%/18.75% in normal, 100%/0 in UC and 84%/16% in CD samples. None of the patients participating in this study suffered from any other known inflammatory or tumorous disease.

For tissue microarray analysis (TMA), 25 formalin-fixed and paraffin-embedded deep tissue samples (10 active UC, 6 active CD and 9 healthy) from the 32 patients involved in the gene expression array investigation, and 45 deep tissue samples (8 active UC, 16 active CD and 21 healthy) of one additional set of patients were used.

Furthermore, we measured the expression levels of 20 selected genes in independent peripheral blood samples (10 healthy and 5 IBD). The diagnostic groups and the number of patients in each group can be seen in Table 1.

2.2. mRNA expression microarray analysis

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) for biopsy samples and the Paxgene Blood RNA Kit (Qiagen) for peripheral blood samples according to the manufacturer's instructions. The isolated peripheral blood RNA samples were concentrated using the GeneChip Blood RNA concentration Kit (Affymetrix Inc., Santa Clara, US). Quantity and quality of the isolated RNA were tested by measuring the absorbance and agarose gelelectrophoresis or capillary gelelectrophoresis using the 2100Bio-analyzer and RNA 6000 Pico Kit (Agilent Inc, Santa Clara, US). Biotinylated cRNA probes were synthesized from 5–8 μg total RNA and fragmented using the One-Cycle Target Labeling and Control Kit (www.affymetrix.com/support/downloads/manuals/expression_s2_manual.pdf) according to the Affymetrix instructions. In case of peripheral blood RNA samples, 5 μg total RNA was used for cRNA probe synthesis, and during reverse transcription Globin Reduction peptide nucleic acid oligomers (Applied Biosystems, Foster City, US) were applied in order to reduce the amount of globin transcripts. Ten μg of each fragmented cRNA sample was hybridized into HGU133 Plus2.0 array (Affymetrix) at 45°C for 16 hours. The slides were washed and stained using Fluidics Station 450 and an antibody amplification staining method according to the manufacturer's instructions. The fluorescent signals were detected by a GeneChip Scanner 3000.

2.3. Statistical evaluation of mRNA expression profiles

2.3.1. Pre-processing and quality control

Quality control analyses were performed according to the suggestions of The Tumour Analysis Best Practices Working Group [36]. 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. The Affymetrix expression arrays were pre-processed by gcRMA with quantile normalization and median polish summarization. The datasets are available in the Gene Expression Omnibus databank for further analysis (<http://www.ncbi.nlm.nih.gov/geo/>). Forty three microarrays (11 normal, 21 IBD biopsy samples and 11 normal blood samples) had been hybridized earlier, their data files were used in previously published studies applying different comparisons [37] and are also available in the Gene Expression Omnibus database (series accession number: GSE4183, GSE10714, GSE10715). The datasets of the newly hybridized 17 microarrays (from peripheral blood samples of 5 patients with CD, 6 patients with UC, 4 diverticulitis, infective colitis and 1 ischemic colitis patients) are registered on the GSE11499 and GSE21826 serial accession number.

2.3.2. Further analyses

To identify differentially expressed features Significance Analysis of Microarrays [38] (<http://www-stat.stanford.edu/~tibs/SAM/sam.pdf>) was used. The nearest shrunken centroid method (PAM) was applied for sample classification from gene expression data [39]. The pre-processing and analysis was performed using R-environment [40] with Bioconductor libraries (<http://bioconductor.org>) [41]. To evaluate the performance of the binary classifier ROC analysis was used maximizing the AUC.

2.4. Real time RT-PCR

For validating the Affymetrix gene expression array method, Taqman real-time PCR was used to measure the expression of 18 selected (all upregulated) genes in case of blood samples and 17 selected (16 up- and 1 downregulated) genes in case of biopsy samples using an Applied Biosystems Micro Fluidic Card System. The measurements were performed using an ABI PRISM® 7900HT Sequence Detec-

Table 1
Number of patients included in the study according to disease groups

Group	Biopsy samples $n = 32$			Independent set of biopsy samples $n = 45$	Blood samples $n = 28$		Independent set of blood samples $n = 15$
	Affymetrix microarray	Taqman RT-PCR	Tissue microarray		Affymetrix microarray	Taqman RT PCR	
Normal	11	5	9	21	11	8	10
Active UC	12	7	10	8	6	2	1
Active CD	9	1	6	16	5	3	4
Diverticulitis	0	0	0	0	4	4	0
Infective colitis	0	0	0	0	1	1	0
Ischemic colitis	0	0	0	0	1	1	0
Total patient numbers	32	13	25	45	28	19	15

tion System as described in the products User Guide (<http://www.appliedbiosystems.com>). In case of both blood and biopsy samples, the selected genes belonged to the maximum discriminatory transcript sets identified by prediction analysis of microarrays method in the IBD versus inflammatory control and normal comparison, and were validated by commercially available Taqman assays. The reactions were performed in triplicates. For data analysis the SDS 2.2 software was used. The extracted delta Ct values (which represent the expression 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.

Triplicated real time RT-PCR was also used in order to examine the expression of channel kinase 2 (TRPM6) and matrix metalloproteinase-9 (MMP9) genes both in blood and in biopsy samples. Beta-actin and 18S ribosomal RNA were used as reference genes. One μg of the isolated total RNA was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Real-time PCR was performed using Light-Cycler DNA Master SYBR Green I (Roche) and newly designed TRPM6, MMP9, beta-actin, and 18S rRNA primers. The sequences of the applied primers were the following: TRPM6: forward: 5'-GTG CGG ACA TGG CAT AAA ATC TTC C-3'; reverse: 5'-CTT CCA GGA ACC TTG GTG TGT AGG-3'; MMP9 forward: 5'-TAC GGC CAC TAC TGT GCC TTT GAG-3'; reverse: 5'-GAG AAT CGC CAG TAC TTC CCA TCC-3'; Beta-actin forward: 5'-GCC AGA GGC GTA CAG GGA TA-3'; reverse: 5'-CGC GAG AAG ATG ACC CAG ATC ATG-3'; 18S rRNS forward: 5'-GTT GAT CCT GCC AGT AGC ATA TGC TTG TCT C-3'; reverse: 5'-AAG GGG GTCA GCG CCC GTC GGC ATG-3'.

After enzyme activation at 95°C for 10 min, 45 PCR cycles were performed (denaturation at 95°C for 10 sec,

annealing at 62°C for 10 sec and extension at 72°C for 20 sec) which was followed by a melting curve analysis.

2.5. Immunohistochemical analyses

2.5.1. Tissue microarray analysis

Cores of 1mm diameter were collected from selected areas of formalin-fixed, paraffin-embedded tissue blocks made from 32 active CD, 40 active UC and 62 normal colon samples of 70 patients by repeating each sample at least once and were then placed into 60 samples recipient blocks. 5 μm thick tissue sections were cut from the TMA blocks, mounted on adhesive glass slides and immunostained following endogenous hydrogen peroxidase blocking (0.5% H_2O_2 -methanol) and heat-induced epitope retrieval in 150 ml of pH 6.0 TRS buffer (Target Retrieval Solution, S1699) using a commercial microwave oven at 300 W power for 45 min. Based on the mRNA expression results some upregulated or downregulated gene products were targeted in TMAs.

TMA slides were incubated with polyclonal goat anti-human matrix metalloproteinase-9 (MMP9, 1:200, AF911, R&D Systems, Minneapolis, USA), Toll-like receptor 5 (TLR5, 1:250, ab1654, Abcam, Cambridge, UK) and guinea pig anti-transient receptor potential cation channel member 6 (TRPM6, 1:100, ab47017, Abcam) for 60 min. Then slides of MMP9 and TLR5 were treated with rabbit anti-goat immunoglobulins (1:200, E0466) for 30 min. The slides treated with TRPM6 were incubated with HRP-conjugated rabbit anti-guinea pig immunoglobulins (1:100, E0466) for 40 min. MMP9 and TLR5 treated slides were finally treated with an anti-rabbit EnVision polymer-HRP conjugate kit (K4003) also for 40 minutes. Peroxidase activity was revealed with a DAB+ substrate chromogen system for 20 minutes followed by hematoxylin counterstain and mounting with by the Faramount medium.

All incubations were carried out in a dark humidified chamber at room temperature and all reagents except where otherwise indicated were obtained from Dako (Glostrup, Denmark).

2.5.2. Immunolabelling on blood smears

The MMP9 immunostaining protocol was carried out on peripheral blood smears of 5 active UC, 5 active CD, 4 diverticulitis, 1-1 infective- and ischemic colitis patients and 5 healthy controls. The blood smears were fixed in -20°C acetone for 5 min. After endogenous peroxidase- and non-specific blocking, and omitting antigen retrieval, MMP9 immunohistochemistry was carried out as described above. All real-time PCR and real-time RT-PCR were performed taking the points of MIQE guidelines into consideration [42].

2.5.3. Fluorescent immunolabeling on blood smears

The TRPM6 and TLR5 immunostaining protocols were also carried out on peripheral blood smears of 5 active UC, 5 active CD, 4 diverticulitis, 1-1 infective- and ischemic colitis patients and 5 healthy controls, which were fixed in acetone -20°C for 5 min. After incubation of smears with TRPM6 and TLR5 immunoglobulins (1:250) for 60 min the glass slides were treated with an Alexa Fluor 488 F(ab')₂ fragment of goat anti-mouse IgG (H+L) (1:200, A11017, Invitrogen) for 30 min. Hoechst Trihydrochloride 0.01% (B-2883; Sigma) was used for staining cell nuclei and the slides were finally mounted by using Faramount (Dako).

2.6. Virtual microscopic evaluation

Immunostained TMA slides and blood smears were digitalized using a high-resolution Mirax Desk instrument (Zeiss, Goettingen, Germany) and the stained spots were analyzed with the Mirax TMA Module software (Zeiss). Protein expression was evaluated using an empirical scoring considering both intensity and frequency in epithelial/lamina propria cells by giving -2 for negative staining; 0 for weak, 1 for moderate and 2 for intensive strong immunostaining. Pearson's Chi-square test and Fischer exact test were performed for the statistical evaluation of marker expression in relation to disease progression. From the two categorical variables (group and score) contingency tables were constructed. To measure the association of two variables the Chi-square test statistic – the sum of the squared Pearson residuals – was used. When the difference was statistically significant ($p < 0.05$), more detailed analysis was

visualized on the basis of the Pearson residuals. These results were summarized in a graphical association plot (<http://gap.stat.sinica.edu.tw/Software/GAP>).

For semi-quantitative counting of immunopositive lamina propria cells 5 fields of view were counted at 200x magnification in each sample core, then means and standard deviation (SD) were determined.

In case of peripheral blood smears, 30 fields of view with 100 μm diameter were evaluated in every sample and the number of immunopositive PBLs (means and SD) was determined.

2.7. Informed consent and approval by the Ethics Committee

All patients included in this study consented to additional research biopsies and peripheral blood samples taken prior to endoscopy. The procedures in the study protocol were approved by the Ethics Committee of the Medical Faculty of Semmelweis University prior to the start of the study.

3. Results

3.1. Gene expression alterations in peripheral blood

Based on the gene expression array results, using PAM and ROC analysis, gene sets including a maximum and a minimum number of transcripts with the same highest specificity and sensitivity values were determined between every disease group comparisons (i.e.: IBD /UC+CD/ vs. non-IBD colitis /diverticulitis+ischemic colitis+infective colitis/ vs. normal controls). The determination of the maximum discriminatory gene set was necessary for further microarray method validation, as there are more Affymetrix transcripts than commercially available TaqMan real-time RT-PCR probes.

IBD blood samples could be differentiated from non-IBD colitis controls (diverticulitis and infective colitis) and normal peripheral blood samples by 100% specificity and 100% sensitivity using a maximum of 141 classifiers, and a minimum discriminatory gene set containing 13 transcripts including Toll-like receptor 5 (TLR5), SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 3 (SMARCD3), interleukin 10 receptor beta (IL10RB) or Bcl-2 interacting killer (BIK).

Each IBD subgroup could be discriminated from the non-IBD colitis and healthy samples by 100% speci-

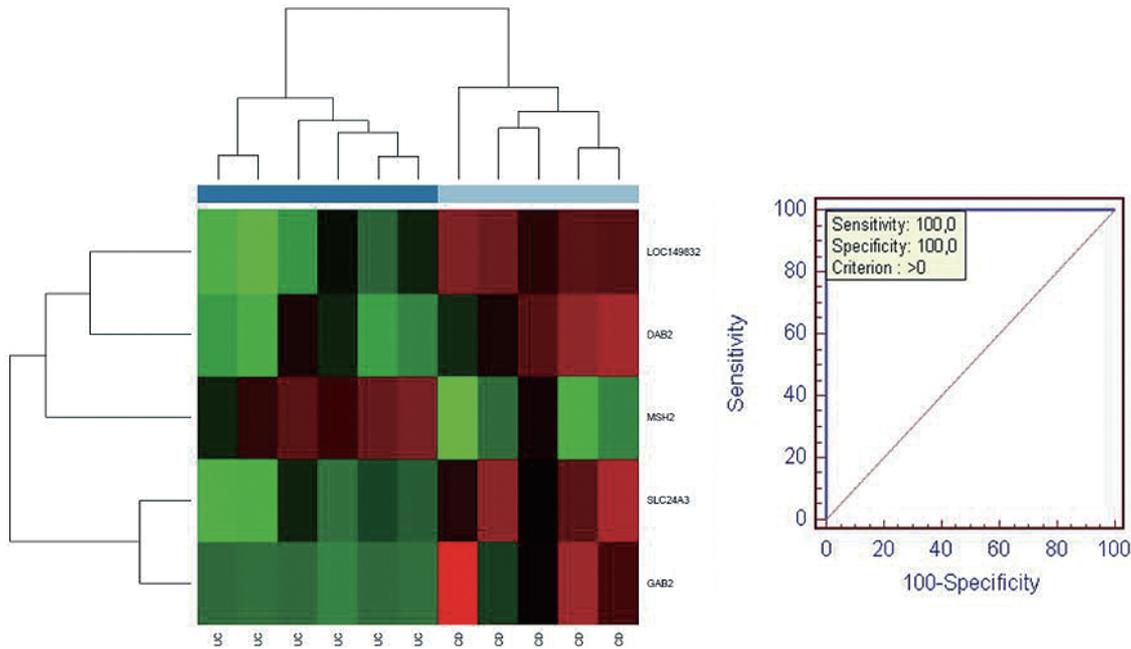


Fig. 1. Heat-map and ROC curve of the minimum discriminatory transcripts in peripheral blood: ulcerative colitis vs. Crohn's disease comparison. Heat-map x-axis: uc: ulcerative colitis; cd: Crohn's disease. Red color represents upregulation, green represents downregulation.

ficity and sensitivity with the help of 163/16 (maximum/minimum) transcripts in UC (e.g.: tumor protein p53 inducible protein 3 /TP53I3/, IL10RB, SMARC3, TLR5) and 99/4 (maximum/minimum) transcripts in CD (e.g.: G protein alpha-inhibiting 2B /GNAI2/, Calcium channel /CACNA1E/).

Using the expression values of 136/5 (maximum/minimum) transcripts such as LOC149832, solute carrier (SLC24A3), disabled homolog 2 mitogen-responsive phosphoprotein (DAB2), GRB-associated binding protein 2 (GAB2) and mutS homolog 2 colon cancer nonpolyposis type 1 (MSH2), significant discrimination could be done between UC and CD with 100% sensitivity and specificity (Fig. 1).

The functional classification of up- and downregulated discriminatory genes is visualized in Fig. 2.

3.2. Gene expression alterations in biopsies

IBD and normal biopsies could be distinguished by 100% sensitivity and specificity using the expression values of 122/3 (maximum/minimum) transcripts including regenerating islet derived /REG/1A, matrix metalloproteinase/MMP/-3, or chitinase 3-like-1/CHI3L1/.

UC samples can be distinguished from normal controls according to the expression of 125/4 (max-

imum/minimum) transcripts (i.e.: CHI3L1, MMP3, SLC6A14, REG1A), while 139/3 (maximum/minimum) transcripts (i.e.: REG3A, REG1B, REG1A) are necessary in order to discriminate between CD and healthy samples with 100% specificity and sensitivity.

UC and CD cases were differentiated by the expression results of 115/58 (maximum/minimum) transcripts by 100% sensitivity and 77.78% specificity.

The functional classification of up- and downregulated discriminatory genes is visualized in Fig. 3.

3.3. Comparison of the direction of gene expression alterations in biopsies and blood samples

Six genes (MMP9, endothelial cell growth factor 1, IL15Ra, ankyrin repeat domain 22, PACAP, LOC440607) showed co-directional, 1 gene (TRPM6) showed anti-parallel expression alteration in IBD blood samples compared to biopsies.

3.4. Validation of gene expression microarray results

3.4.1. RT-PCR results

9 of the 18 selected upregulated genes measured by the Taqman method significantly correlated with the expression results obtained by Affymetrix microarrays ($p < 0.05$) comparing IBD vs. normal and non-IBD

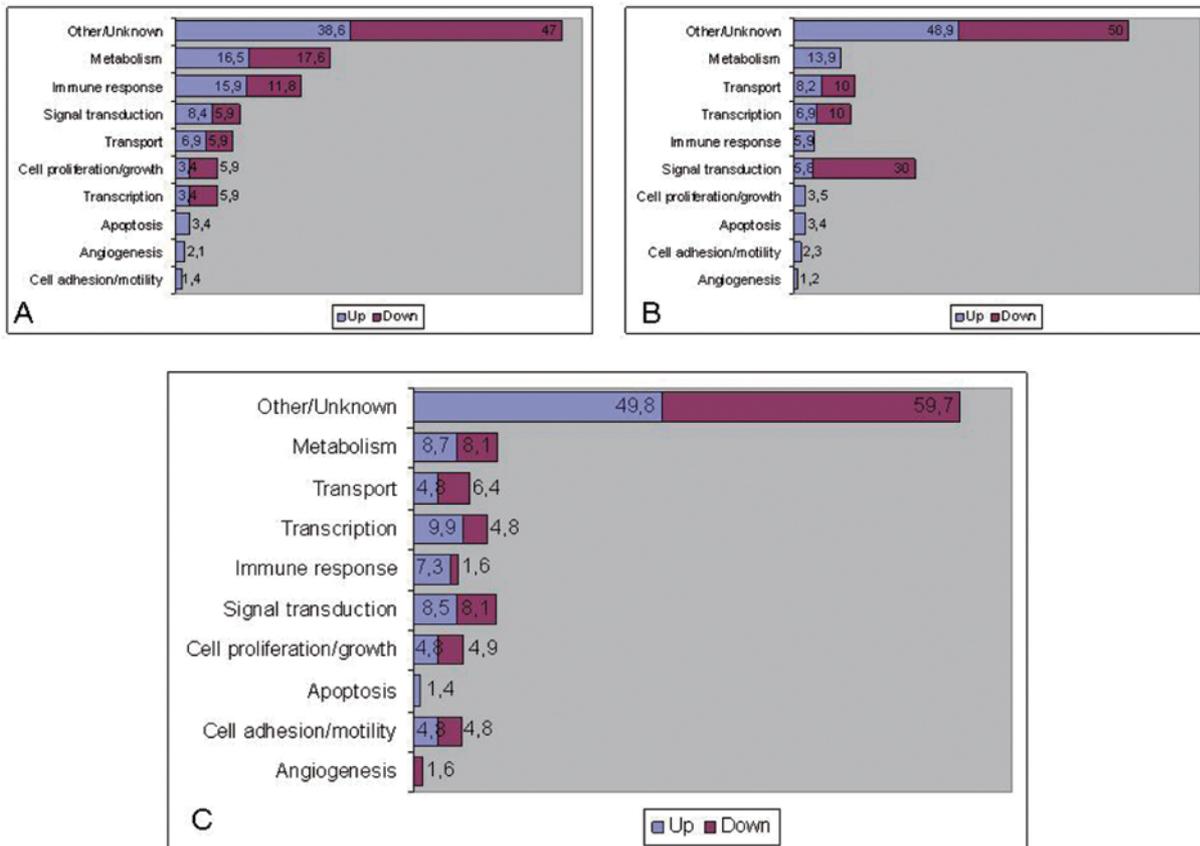


Fig. 2. Functional classification of discriminatory genes in peripheral blood. A: UC vs. non-IBD and normal controls; B: CD vs. non-IBD and normal controls; C: UC vs. CD blood samples. Gene functions are represented on the y-axis. The numbers in the bars represent the percentage of genes belong to the given functional category.

control peripheral blood samples. In case of the other 9 genes strong tendency was found in the expression results.

The expression of all selected (16 upregulated and 1 downregulated) genes measured by Taqman RT-PCR significantly correlated with the expression results obtained by Affymetrix microarrays ($p < 0.05$) comparing IBD vs. normal biopsy samples. Taqman quantitative comparison was done between 8 IBD (7 UC and 1 CD) and 5 normal tissue biopsies, because only 1 RNA sample has remained from the CD samples after microarray analysis.

The cycle variation of the same samples between the triplicated Taqman RT-PCR reactions was under 0.5 cycles in every case. The results of the Taqman RT-PCR validation on selected genes and the connection between RT-PCR and Affymetrix results are represented in Figs 4 and 5.

According to the microarray results, significantly elevated MMP9 mRNA expression was found in periph-

eral blood samples of patients with IBD compared to non-IBD and healthy controls ($p < 0.05$). This alteration was also detectable in IBD biopsy samples using both Taqman and a LightCycler assay.

While TRPM6 anti-parallel expression in biopsy and blood samples was measured using microarrays, this phenomenon could not be validated by RT-PCR. However, significantly decreased TRPM6 mRNA levels were detected by Taqman RT-PCR in IBD biopsy samples compared to healthy controls.

3.4.2. Immunohistochemistry

MMP9 was detected in peripheral blood smears from active IBD patients. The number of MMP9 positive PBLs was significantly higher in active UC (14.6 ± 0.9 cells/ field of view) and CD (15.6 ± 0.6 cells/ field of view) than in normal samples (1.2 ± 0.4 cells/ field of view) or non-IBD controls (1.8 ± 0.6 cells/ field of view) ($p < 0.005$).

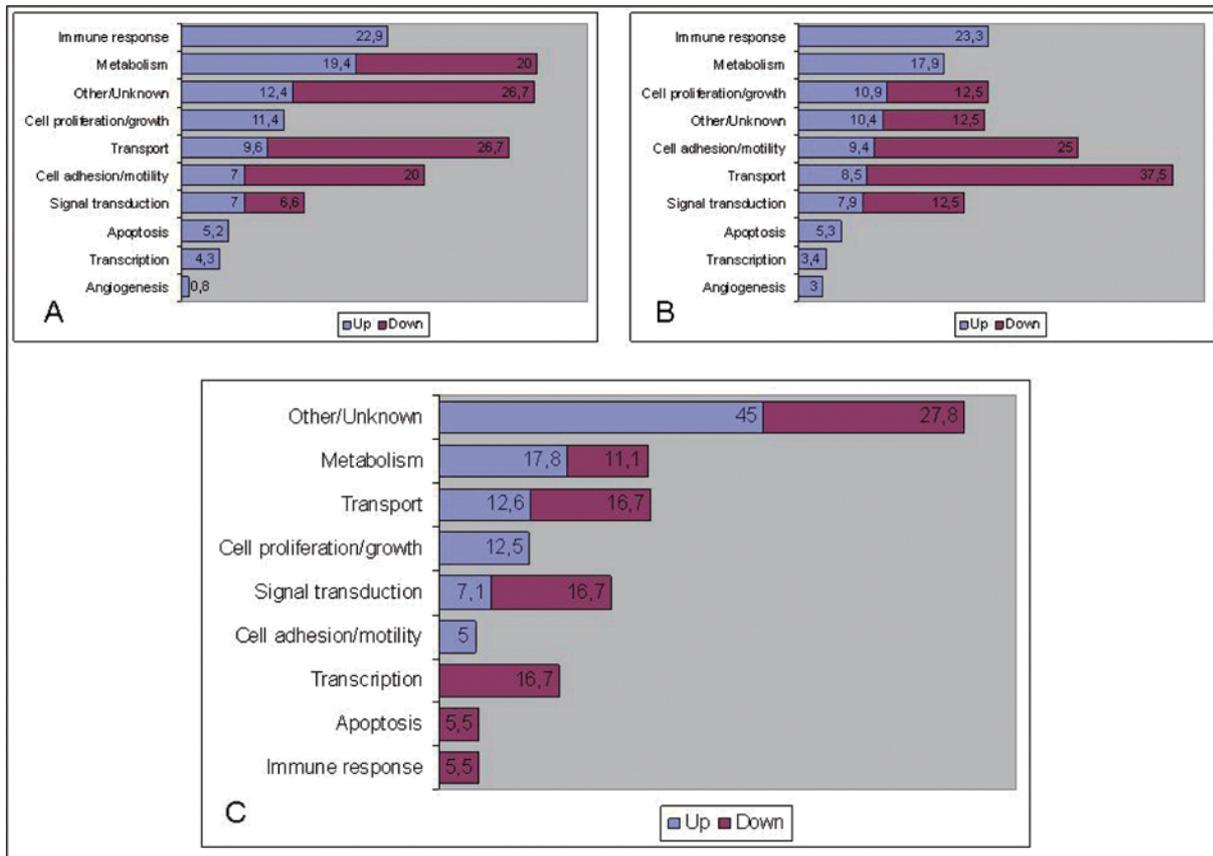


Fig. 3. Functional classification of discriminatory genes in biopsies. A: UC vs. normal; B: CD vs. normal; C: CD vs. UC biopsy samples. Gene functions are represented on the y-axis. The numbers in the bars represent the percentage of genes belong to the given functional category.

MMP9 immunohistochemistry in biopsies also significantly correlated with the gene expression analysis results. MMP9 expression significantly increased in inflamed colonic mucosa compared to normal samples (UC: 49.4 ± 2.9 cells/field of view; CD: 52.7 ± 3.3 cells/field of view; normal: 9.5 ± 1.9 cells/field of view; $p < 0.001$). Although MMP9 epithelial expression showed an anti-parallel increase in expression, cumulative MMP9 expression (epithelium and lamina propria together) significantly correlated with the microarray results.

TLR5 was also detected in peripheral blood smears from active IBD patients. The number of TLR5 positive PBLs was significantly higher in active UC (28 ± 1.4 cells/ field of view) and CD (32 ± 1.1 cells/ field of view) than in normal samples (1.4 ± 0.6 cells/ field of view) ($p < 0.005$) or inflammatory controls (2.9 ± 0.7 cells/ field of view) ($p < 0.005$).

In peripheral blood smears, TRPM6 positive PBLs were only seen rarely in normal (1 ± 0.3 cell/ field of view) and non-IBD (1.7 ± 0.8 cell/ field of view)

controls compared to active IBD samples (UC: 11.4 ± 1.4 cells/ field of view; CD: 14.6 ± 0.8 cells/ field of view) ($p < 0.05$). All p-values represent differentiation between UC/CD samples and normal controls.

TLR5 and TRPM6 immunohistochemistry results also significantly correlated with the gene expression array results. The number of TLR5 lymphocytes in active UC and CD lamina propria was significantly higher compared to the normal cases (UC: 102 ± 5 cells/ field of view; CD: 94.5 ± 6.3 cells/ field of view; normal: 4.7 ± 1.4 cells/ field of view; $p < 0.001$).

The anti-parallel expression of TRPM6 observed by gene expression microarray analysis could be validated by immunohistochemistry. The number of TRPM6 positive lymphocytes was significantly higher in normal colon (90.5 ± 5.5 cells/ field of view) than in active UC (8.3 ± 1.9 cells/ field of view) and active CD (13.2 ± 1.5 cells/ field of view) ($p < 0.001$). The results of MMP9, TLR5 and TRPM6 immunohistochemistries can be seen in Figs 6 and 7.

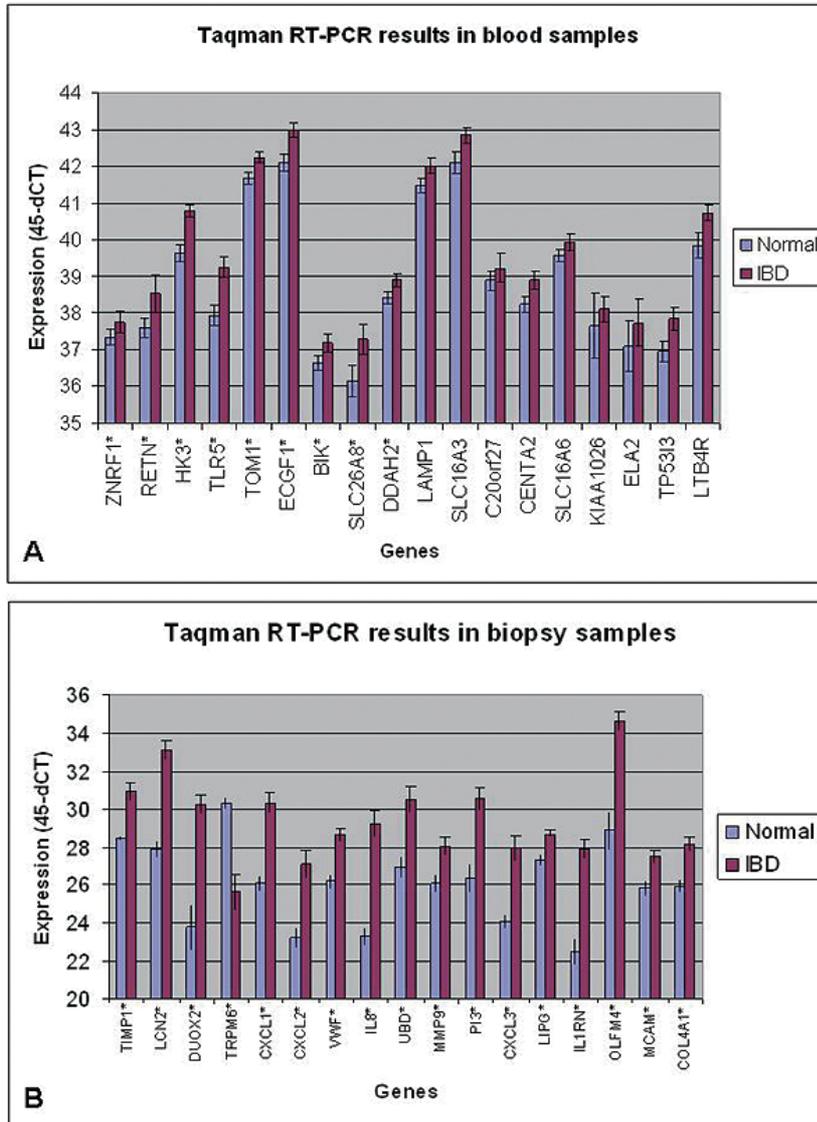


Fig. 4. The results of the Taqman RT-PCR validation on selected genes. A: Peripheral blood samples; B: Biopsy samples. (Means of expression values (45-dCT) and scatters; genes with significant expression alterations at $p < 0.05$ level are marked with *).

4. Discussion

In this study the gene expression profile of peripheral blood samples of patients with IBD, non-IBD colitis (namely diverticulitis, infective colitis and ischemic colitis) and of healthy individuals were compared in order to find discriminative gene expression alterations that could be used for diagnostics and screening of IBD. Some of the discriminative transcripts could be identified in the inflamed colon, as well.

It is a fact that the members of the circulating immune system play a messenger role between tissue spe-

cific alterations and peripheral blood [33,43–45]. On the other hand, based on the immune surveillance characteristic of peripheral blood cells the source of gene expression profile alterations detected in them could not be originated just one distinct local disease process. Therefore, in order to enhance the specificity of screening and diagnostic assays, the identification of local tissue and disease specific information in peripheral blood is of great importance.

Using genome-wide gene expression analysis combined with PAM, discriminant and ROC analyses, such a discriminative gene expression pattern with the

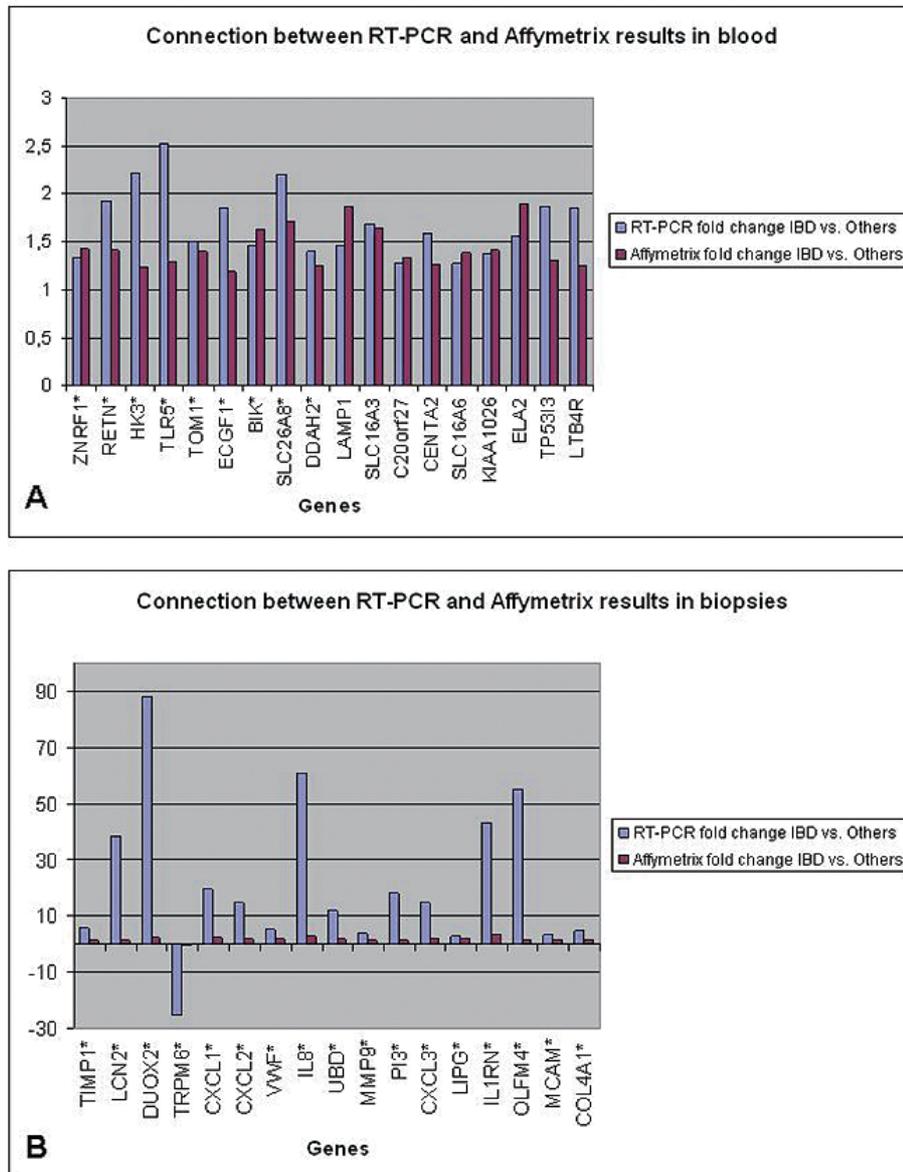


Fig. 5. The connection between RT-PCR and Affymetrix results. A: peripheral blood samples; B: biopsy samples. The differences of the means of expression values /45-dCT/ between IBD vs. other cases (normal and non-IBD controls) and the Affymetrix foldchange values are visualized; genes with significant expression alterations at $p < 0.05$ level are marked with *).

range containing maximum and minimum number of transcripts may be determined which can be disease-specific. Based on the characteristic features of the used analytical techniques, this pattern consists of genes that may be different from the genes with the highest expression alterations.

In peripheral blood samples, we detected 141 transcripts that showed significant expression alteration between IBD patients, non-IBD colitis cases and healthy

subjects. Using the same peripheral blood samples we were able to determine a set of 136 transcripts that can differentiate between UC and CD by 100% specificity and sensitivity. Moreover, the top 5 transcripts could be identified by which a significant discrimination can be performed between the two conditions without a decrease of specificity and sensitivity. Some of these are such genes that are known to play an important role primarily in inflammatory and tumorous processes, others

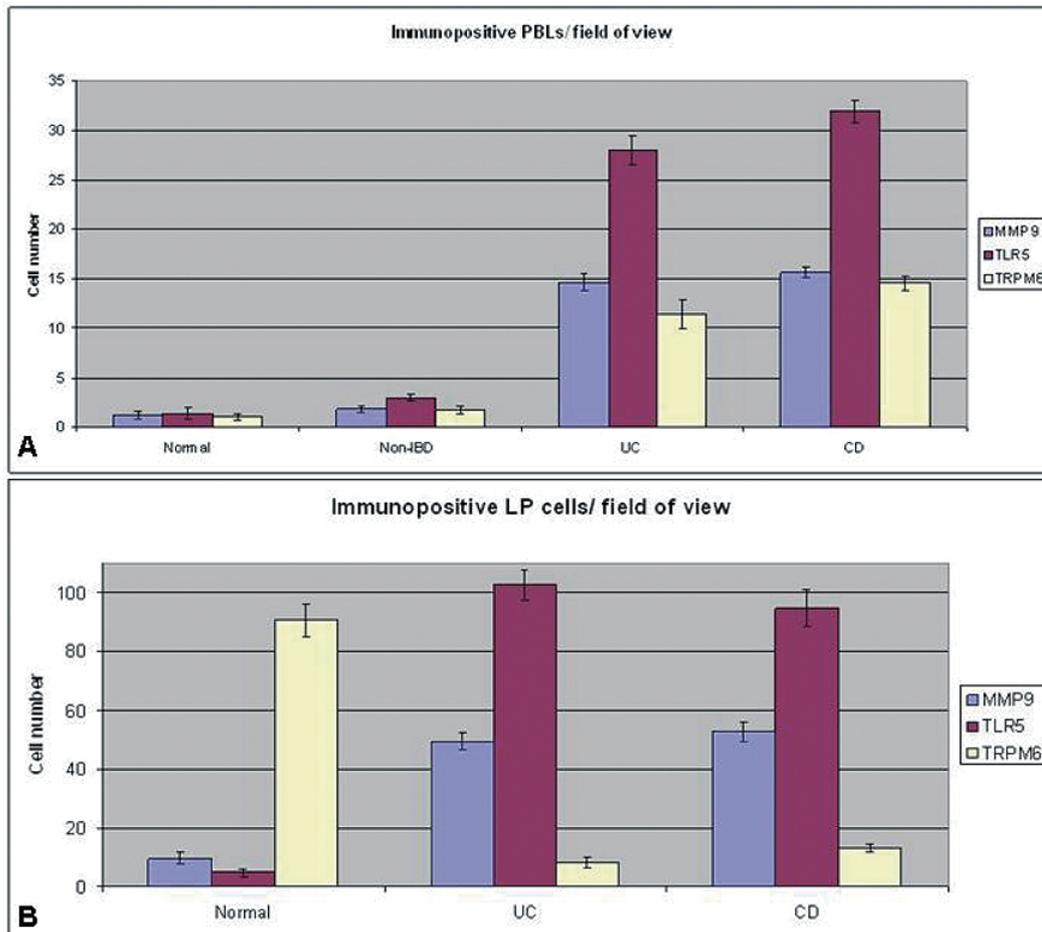


Fig. 6. Graphical visualization of the immunohistochemical results. The y-axis represents the number of MMP9, TLR5 and TRPM6 immunopositive peripheral blood leukocytes (PBLs; A) and lamina propria cells (LP; B) per field of view in UC, CD, non-IBD cases and normal controls (means and scatters).

have unknown biological functions.

MMP9, a metalloproteinase involved in tumor invasion [46] was found to be overexpressed in blood samples of IBD patients. Its serum level was showed to be a predictive factor in lung cancer [47]. Whether the high number of MMP9 positive PBLs is just an inflammation related phenomenon in the peripheral blood, or whether these cells have a specific role in the circulation is still unknown. The fact that MMP9 was found to be overexpressed in IBD but not in non-IBD cases suggests that it has a special role in the pathogenesis of IBD that needs to be further studied.

Toll-like receptor 5 is a member of the Toll-like receptor family which plays a fundamental role in pathogen recognition and activation of innate immunity [48]. TLR5 is expressed in myelomonocytic cells and recognizes bacterial flagellin, a principal compo-

nent of bacterial flagella and a virulence factor. The activation of this receptor mobilizes the nuclear factor NF-kappaB and stimulates tumour necrosis factor-alpha production [48,49,51]. A strong serological response to flagellin in multiple animal models of colitis has been reported by Lodes et al. [52] who also proved that colitis can be induced by transferring flagellin-specific T cells to immunodeficient animals. Further results implicating TLR5 polymorphisms in the pathogenesis of inflammatory bowel disease suggests that carriage of a dominant negative TLR5 polymorphism appears to protect against Crohn's disease and results in a significant reduction in flagellin-specific circulating IgA and IgG concentrations [53]. These data linking a genetic defect in TLR5 to an alteration in the acquired immune response are interesting and of great pertinence as recent studies report synergism be-

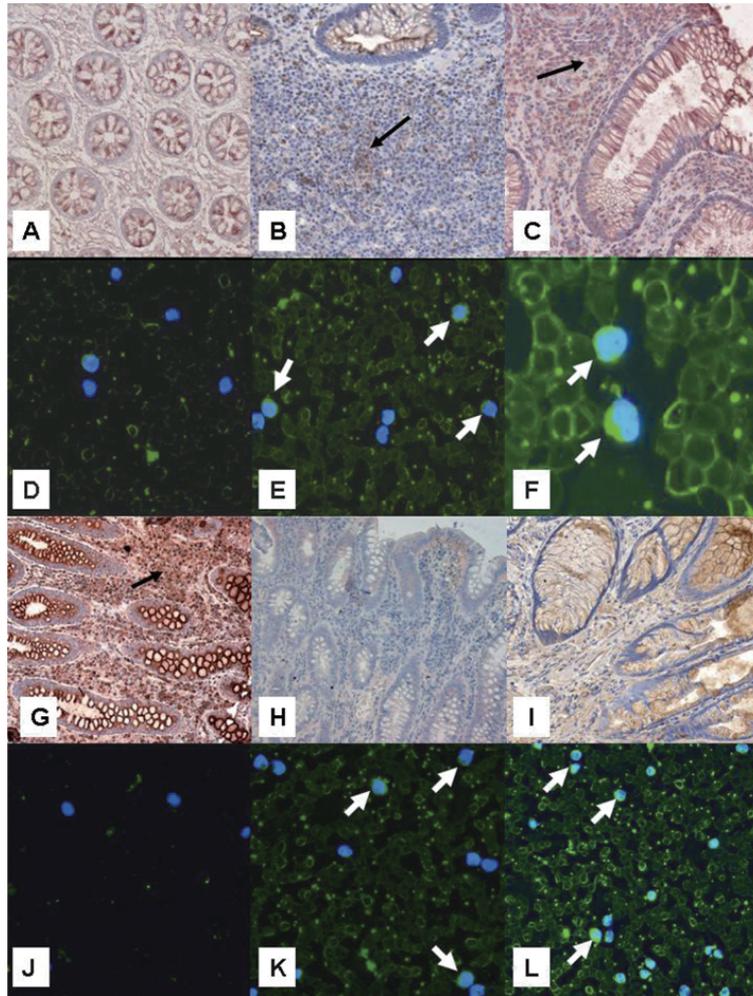


Fig. 7. The results of TLR5 (A-F) and TRPM6 (G-L) immunohistochemistries. A, G: normal colon (black arrow: TRPM6 positive lamina propria lymphocytes) (180x magnification); Active CD (B, H) and active UC (C, I) (black arrow: TLR5 positive lamina propria lymphocytes) (A: 180x, B: 200x, C: 200x, G: 180x, H: 180x, I: 200x magnification); TLR5/TRPM6 positive lymphocytes in peripheral blood smears: normal controls (D, J), active CD (E, K), active UC (F, L) (white arrows) (D: 200x, E: 190x, F: 700x, J: 350x, K: 330x, L: 450x magnification). Images were taken using a virtual microscope. Note: although the scale of magnification by virtual microscope may be varied from 0 to 1000x, or even more, this scale is not continuous, and may be influenced by several technical factors (for more information visit www.3dhitech.com).

tween NOD2/CARD15 and TLR5 signalling [54]. In our microarray study, we found TLR5 overexpressed only in blood samples of active IBD patients but no significant TLR5 expression alteration was detected in biopsy samples. As the involvement of TLR5 positive lymphocytes in IBD pathogenesis is uncertain without their confirmed presence in the colon, we decided to examine TLR5 protein expression in biopsies. The immunohistochemistry results proved their presence in the inflamed lamina propria, especially in subepithelial lymphoid aggregates, which is known to be a switching point between circulation and colonic mucosa [55]. The deep subepithelial location of TLR5 positive cells

may be an explanation of the low TLR5 mRNA expression in biopsies containing mainly epithelial layer with adjacent lamina propria.

Some gene expression alterations showed different directions in blood samples and biopsies which may be explained by either primary and/or secondary immunological processes taking place in the inflamed colon. In primary immune processes the mucosal homing of immune cells results in a decrease of peripheral signals. On the other hand, the parallel increase of immune related signals (e.g: TLR5, MMP9) in both colonic tissue and blood samples may represent a secondary immune process that is mainly connected to CD45+RO (mem-

ory) T-cells. The behaviour of TRPM6 positive leukocytes supports none of the above mentioned hypotheses. We showed TRPM6 to be expressed by PBLs in active IBD, however, the function of this cation channel is still unknown in leukocytes. The behavior of TRPM6 positive PBLs is intriguing as their number is increased in peripheral blood in case of active IBD (but not in non-IBD controls) and decreased in the inflamed mucosa which suggests that this type of leukocyte may change its phenotype in an inflammatory environment, such as IBD. Further characterization of these cells seems to be interesting in order to elucidate their role in IBD pathogenesis.

Using normal biopsy samples for whole genomic gene expression analysis, 122 transcripts were found that showed significant expression alteration between IBD and normal, healthy colon. Using the same biopsy samples, we were able to determine gene sets that can differentiate between the main subgroups of IBD, UC and CD. These methods may also be an important tool in enhancing the efficacy of conventional diagnostic methods like histology in case of undetermined colitis which could be up to 10% of IBD cases.

In biopsy samples such candidate genes were identified among others that are known to have important roles in inflammatory and tumorous processes.

The REG genes are known to inhibit calcium carbonate crystal growth in the human pancreas and kidney as well [56–58]. REG1a was shown to have a potential role in the pathogenesis of gastrointestinal inflammations such as *Helicobacter pylori* induced gastritis [59], gastric ulcer lesions [60,61], and pancreatitis [62,63]. In accordance with the results of Shinozaki et al. [64] and Dieckgraefe BK et al. [32] we think that the REG protein family may be involved in the pathogenesis of the described epithelial cell proliferation and apoptosis disturbance in active IBD and works as a regenerative factor [65].

It has been proposed that matrix metalloproteinases (MMPs) play a role in tumor invasion, and we recently reported the overexpression of MMP9 to be strongly associated with colorectal carcinogenesis in the dysplasia-adenoma-carcinoma sequence [46]. MMP9 is mainly involved in tissue remodeling in IBD [66]. In this study the amount of MMP9 expression was found to be increased in lamina propria in relation to inflammation. Moreover, we confirmed the immunohistochemical results reported by Gao et al. [67] showing that the prominent location of MMP9 is in the submucosa. In addition, a pronounced positive staining for MMP9 in polymorphonuclear leukocytes was observed

which was found to increase parallel with the severity of inflammation. Furthermore, the polymorphonuclear leukocytes were primarily identified as the main source of MMP9 within the inflamed colon with occasionally positive macrophages whereas myofibroblasts were found to be MMP9 negative, the latter has also been confirmed by *in vitro* studies [68]. Although, the pathogenetic role of MMP9 in IBD needs further investigations, the extensive presence of MMP9 in both the inflamed tissue and peripheral blood makes this protein a promising candidate for targeted biological therapy.

The discovery of the channel kinase 2, also known as transient receptor potential cation channel member 6 (CHAK2/TRPM6) originates in the studies of Schlingmann et al. [69] and Walder et al. [70]. Both research groups studied hypomagnesemia with secondary hypocalcemia and proved this condition to be due to the mutations in TRPM6. TRPM6 mRNA has been detected in colon epithelial cells, duodenum, jejunum, and ileum [69]. According to our knowledge, TRPM6 has never been described in association with inflammatory bowel diseases before [71]. The TRPM6 down-regulation in colonic tissue described in our study may contribute to the symptoms associated with hypomagnesemia in IBD [72–74].

By comparing the GEP results of peripheral blood and biopsy samples, we detected for the first time ECGF1, IL15RA, ankyrin repeat domain 22, PACAP, LOC440607 (hypothetic protein with unknown function), and TRPM6 as possible active IBD-associated transcripts that can also be detected in the inflamed colonic tissue. By using parallel comparison of peripheral blood and diseased colonic tissue, the discovery of tissue specific disease-related circulating transcripts could be more accurate than only focusing on blood alone.

Comparing our results to the annotated 12 UC vs. CD classifier genes by Burczynski et al. [33] there is only one similarity, granzyme K, a serine protease, also known as granzyme 3 which we also found to be upregulated in UC compared to CD in this study. On the other hand, we observed chemokine (C-X-C motif) receptor 5 to be overexpressed in PBLs of UC patients while Burczynski et al. described chemokine (C-X-C motif) ligand 5 specific for CD. The slight overlap between transcripts identified in the present set and those reported in the study of Burczynski et al. may be explained not only by the difference in the number of patients investigated by microarray in the two studies (28 in ours vs. 127 in Burczynski's) and the use of non-IBD colitis controls in our study, but it can also

be attributed to different sample processing protocols. For blood sample collection we used Paxgene tubes that conserve the PBLs' mRNA immediately, unlike Burzynski et al. where RNA isolation was performed only after overnight shipping from the place of blood collection to the place of the central processing laboratory. In our study, all involved patients belong to the Caucasian race, in contrast to the other study. Another possible reason for the difference in results may be that we used Affymetrix HGU133 Plus2.0 arrays, the so-called global arrays for gene expression profiling. Moreover, the application of different statistical methods during the evaluation of results in these studies may also explain the varying results. Reviewing the current scientific literature there is yet no consensus as to the statistical evaluation of the enormous amount of microarray data.

Interestingly, the specificity of UC and CD sample discrimination by gene expression profiles in conventional biopsies was lower than in blood samples. One possible explanation of this discrepancy may stem from technical aspects, namely, if the biopsy is taken inaccurately, some biopsies may contain less inflamed mucosa. As we found, some discriminative transcripts were located in deep lymphoid follicles of the diseased colon. Normal biopsies do not always reach this depth. Second, although UC and CD are distinct diseases, the inflammatory processes have several common points that may also result in lower specificity. On the other hand, it is well known that gene expression profiles may be influenced by all kinds of inflammatory processes in peripheral blood. This is why we exclusively included patients with only one known inflammatory disease in this study. Initially, a higher number of IBD patients were examined during this study but most of them were excluded due to other inflammatory and/or benign/malign tumorous diseases such as arthritis, reflux oesophagitis, primary sclerosing cholangitis, colon adenoma or colorectal cancer. It was the aim of our study to include only homogenous groups of patients in order to be able to draw clear-cut conclusions. If the groups of patients included in our study had been non-homogenous, it would have rendered the explanation of our results much more difficult.

In conclusion, using high density oligonucleotide microarrays combined with PAM and ROC analysis we were able to establish a methodology that can be an initial basis for a population based screening study to find a discriminative gene expression pattern for UC and CD cases with high sensitivity and specificity. We also pinpointed for the first time 6 active IBD-associated

transcripts in peripheral blood that can also be detected in the inflamed colon.

Whether they may predict the activity of the inflammation is not clear yet, but using a larger sample set including active and non-active IBD patients may help us to determine genes that can be used as activation markers from peripheral blood. As most of the colonic microarray studies, we also found a lot of genes with unknown function at this time. Further study of them is of great importance. With the help of screening microarray studies in IBD, discriminatory gene sets can be determined and the selected disease-specific genes can later be used in daily routine for conventional RT-PCR methods like Taqman cards or LC480 instruments.

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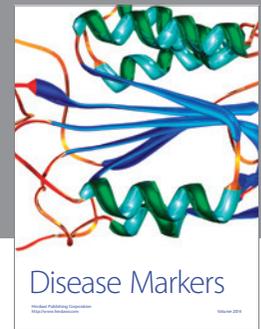
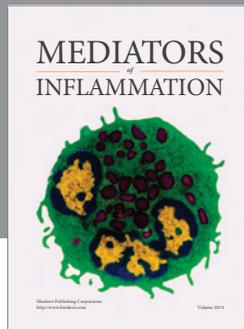
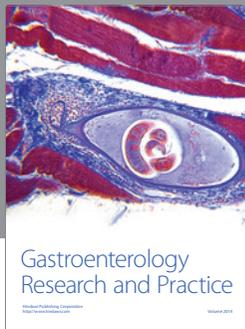
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