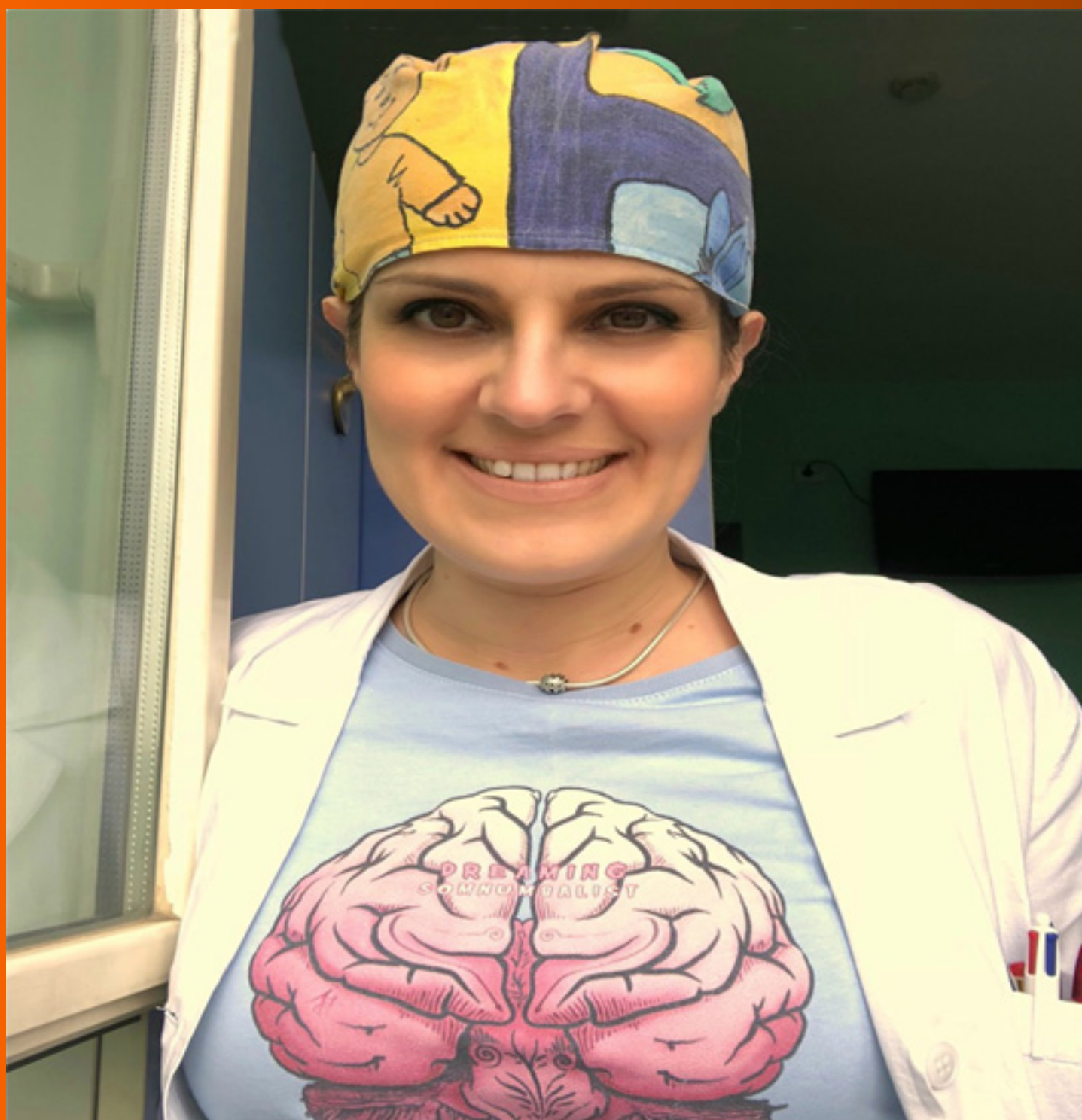


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Observational Study

Multiplex gene expression profile in inflamed mucosa of patients with Crohn's disease ileal localization: A pilot study

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Abstract**BACKGROUND**

Crohn's disease (CD) is a complex disorder resulting from the interaction of genetic, environmental, and microbial factors. The pathogenic process may potentially affect any segment of the gastrointestinal tract, but a selective location in the terminal ileum was reported in 50% of patients.

AIM

To characterize clinical sub-phenotypes (colonic and/or ileal) within the same disease, in order to identify new therapeutic targets.

METHODS

14 consecutive patients undergoing surgery for ileal CD were recruited for this

Torcia MG and Piccinni MP participated in the writing of the study protocol and in the revision of the manuscript for important intellectual content with Ficari F, Tonelli F, Biancone L, Fazi M, Scaringi S and Delfino G. Perissi E and Russo E edited the manuscript. Malentacchi C, Giudici F, Russo E, Piccinni MP wrote the manuscript. Malentacchi C and Russo E provided funding acquisition. All authors read, commented, and approved the final manuscript.

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study. Peripheral blood samples from each patient were collected and the main polymorphisms of the gene *Card15/Nod2* (R702W, G908R, and 1007fs) were analyzed in each sample. In addition, tissue samples were taken from both the tract affected by CD and from the apparently healthy and disease-free margins (internal controls). We used a multiplex gene assay in specimens obtained from patients with ileal localization of CD to evaluate the simultaneous expression of 24 genes involved in the pathogenesis of the disease. We also processed surgery gut samples with routine light microscopy (LM) and transmission electron microscopy (TEM) techniques to evaluate their structural and ultrastructural features.

RESULTS

We found a significant increase of Th17 (IL17A and IL17F, IL 23R and CCR6) and Th1 (IFN- γ) gene expression in inflamed mucosa compared to non-inflamed sites of 14 CD patients. *DEFB4* and *HAMP*, two genes coding for antimicrobial peptides, were also strongly activated in inflamed ileal mucosa, suggesting the overwhelming stimulation of epithelial cells by commensal microbiota. IFN- γ and CCR6 were more expressed in inflamed mucosa of CD patients with ileal localization compared with patients with colonic localization suggesting a more aggressive inflammation process in this site. Morphological analysis of the epithelial lining of Lieberk \ddot{u} n crypts disclosed enhanced release activity from goblet mucocytes, whereas the lamina propria contained numerous cells pertaining to various lines.

CONCLUSION

We observed that the expression of ileal genes related to Th1 and Th17 activity is strongly activated as well as the expression of genes involved in microbiota regulation.

Key words: Crohn's disease; Ileum; Colon; Messenger ribonucleic acid; Th1/Th17; Microbiota; Inflammation

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Core tip: Multiplex Gene Assay in specimens obtained from patients with ileal localization of Crohn's disease (CD) allowed the simultaneous analysis of messenger ribonucleic acid levels for 24 genes, known to be involved in the inflammation processes of CD pathogenesis. The result showed that the expression of genes related to Th1 and Th17 immune response is strongly activated as well as the expression of genes deputized to interact with the commensal microbiota, such as *DEFB4* and *HAMP*, which code for antimicrobial peptides.

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INTRODUCTION

The pathogenesis of Crohn's disease (CD), one of the major inflammatory bowel diseases (IBD) together with ulcerative colitis (UC), has been extensively investigated. It is generally accepted that both genetic and environmental factors contribute to the etiology of the disease. In CD patients, strong associations between genes involved in maintaining intestinal barrier function, epithelial anti-microbial defence, innate immune regulation, reactive oxygen species (ROS) generation, autophagy, and metabolic pathways have been identified^[1,2].

Environmental risk factors involved in the progression of the disease include

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smoking, low-fiber and high-carbohydrate diet, gut microbiota (GM) alteration, and treatments with antibiotics or non-steroidal anti-inflammatory drugs^[3].

CD is characterized by a transmural inflammation which can potentially affect any segment of the gastrointestinal tract^[4]. However, recent studies reported a selective location in the terminal ileum in 50% of the patients and location in the colonic district in 20% of the patients. Ileum and colon district were involved in the remaining 30% of the patients. A different clinical course and surgical requirement was reported according to disease's localization but currently the reasons underlying the differences in the clinical course have not been defined. In addition, the immunological pathways involved in colonic inflammation are different from those involved in ileal inflammation^[5].

The mutual interplay between GM and the immune system is involved in the pathogenesis and prognosis of intestinal diseases^[6] as the GM is a key modulator of intestinal inflammation^[7]. In CD patients, a reduced GM diversity and lower bacterial load in inflamed *vs* non-inflamed tissues was observed^[8]. In addition, several evidences report that the small bowel is responsible for the systemic tolerance towards microbes. A recent study revealed that the ileum harbors a distinctive niche of the GM that differs more from the colonic^[9]. This different GM composition could be attributed to the activation of distinctive immunological pathways.

In the present study, we used the multiplex gene assay^[10,11] to analyze surgical specimens of CD patients with prevalent ileal localization.

MATERIALS AND METHODS

Patients

14 consecutive patients undergoing surgery for ileal CD aged 15 to 57 and hospitalized at the Surgery Unit of Azienda Ospedaliero-Universitaria Careggi, University of Florence, were recruited (Table 1). CD was diagnosed based on both histological and clinical/endoscopic criteria. Table 1 reports the clinical characteristics of the patients.

Peripheral blood samples from each patient were collected in EDTA tubes and genomic DNA was extracted using QIA-AMP DNA Blood Maxi Kit (Qiagen GmbH, Hilden, Germany). The main polymorphisms of the gene *Card15/Nod2* (R702W, G908R, and 1007fs) were analysed in each sample^[12].

mRNA extraction and multiplex Gene Assay

Tissue samples were taken both from the tract affected by CD and from the apparently healthy and disease-free margins (internal controls). The surgical specimens were opened longitudinally.

All samples were stored in RNA later (Qiagen, Germany) before homogenization. Then each sample was weighed and the appropriate lysis solution was added to a final volume of 150 μ L containing 50% Lysis Mixture (Thermo-Fisher, MA, United States) and 1 g/L proteinase. The mixture was agitated for 30 min at 65 °C to lyse the cells. The lysate was stored at -80 °C for later use. We used a microarray panel of 24 genes implicated in CD etiopathogenesis^[10]. We evaluated the expression of these genes in both non-inflamed and inflamed ileal biopsies. Table 2 indicates the panel of the examined genes, the number of Mendelian Inheritance in Man (MIM) (used as a reference), accession number and their corresponding encoded product and function. To improve the analysis of the results, the selected genes were divided into four groups according to their biological role: (1) Transport across epithelia: *ABCB1*, *SLC40A1*, *SLC22A4*, *SLC22A5*, *HAMP*; (2) Immune response: *CCR6*, *IL-17F*, *IL-17A*, *MICA*, *MYD88*, *STAT3*, *IL-23R*, *JAK2*, *IFNG*, *NOD2*; (3) Antimicrobial activity: *HAMP*, *CAMP*, *LRRK2.DEFB4*; and (4) Physiological activities: *STAT3*, *ESR1*, *LRRK2*, *TNFSF15*, *CARD14*, *DLG5* *BMP2* *ATG16L1*.

The messenger ribonucleic acid (mRNA) expression for *CCR6*, *IL-17A*, *IL-17F*, *BMP2*, *TNFSF15*, *ABCB1*, *IL-23R*, *DEFB4*, *CARD14*, *STAT3*, *SLC40A1*, *JAK2*, *SLC22A5*, *ACTB*, *ATG16L1*, *CAMP*, *DLG5*, *ESR1*, *CARD15*, *MICA*, *MYD88*, *SLC22A4*, *IFN- γ* , *LRRK2*, *HAMP*, *ACTB* (high expression housekeeping gene), *HPTR1* (low expression housekeeping gene) was measured using the QuantiGene[®] Plex assay (Thermo-Fisher, MA, United States).

A panel of oligonucleotide capture probes was covalently linked to carboxylated fluorescently encoded beads (Luminex, Bio-Rad, MA, United States). Each probe has a unique sequence of 15 bases. Each sample lysate diluted at 1:1 and 1:2 was mixed with the pooled capture beads in a round-bottom assay well and hybridized for 16 h at 54 °C (final volume in each well was 100 μ L). The assay mixture was moved to a MultiScreen[®] Filter Plate (Millipore, Billerica, MA, United States) and unbound

Table 1 Clinical characteristics of patients with ileal Crohn's disease

Patient	Pt1	Pt2	Pt3	Pt4	Pt5	Pt6	Pt7	Pt8	Pt9	Pt10	Pt11	Pt12	Pt13	Pt14
Localization of CDa	L1	L1	L1	L1	L1	L1	L3	L1	L3	L1	L3-L4	L1	L3	L1
Age of CD onset (yr)	57	15	53	55	25	31	39	42	16	24	19	18	46	30
Surgery / relapse	1st surgery	1st surgery	Relapse	1st surgery	Relapse	Relapse	Relapse	1st surgery	Relapse	Relapse	Relapse	Relapse	Relapse	1st surgery
Disease behaviorb	B2	B2, B3	B2	B3	B2, B4	B2	B3	B2	B2	B2	B2	B2	B3	B2
Therapyc	C, F, I, B	F, C	C	No	F, C, I	C, F, I	I, B	F, C	C, I, F, B	C	F, C, B	F, C, I	C, F, B, I	F, C, I
Smoking statusd	No	Cur	No	No	10/10	No	No	20/30	10/10	Cur	10/20	No	No	No
Genotype	wt	R702	wt	hzG881R	wt	wt	wt	wt	hzG881R	R702	No seq	No seq	wt	wt

Localization of Crohn Disease: L1: terminal ileum; L2: colon; L3: ileum colon; L4: upper G (gastrointestinal); Disease behavior: B1: non-stricturing, non-penetrating; B2: stricturing; B3: penetrating; B4: perianal disease. Therapy: M: mesalazine; I: immunosuppressant; B: biologics; C: corticosteroids; Ab: antibiotics. Smoking status: No: non-smoker; Ex: ex-smoker; Cur: current smoker, no. cigs per day / no. yr. MIM: Mendelian inheritance in Man. 1: Transport across epithelia; 2: Immune response; 3: Antimicrobial activity; 4: Different physiological activities.

material was filter-washed from the wells by rinsing 3 times with wash buffer. The plate was hybridized with 100 μ L/well of bDNA amplifier in Amplifier Diluent (Panomics, CA, United States) at 54 °C for 1 h. After the plate was filter-washed twice with wash buffer and incubated at 50 °C for 1 h with 100 μ L/well of 5'-dT(Biotin)-conjugated label probe (Panomics, CA, United States) diluted in Label Probe Diluent (Panomics, CA, United States). After 2 washes, streptavidin-conjugated R-phycoerythrin diluted in SA-PE diluent (20 mmol/L Tris-HCl, 400 mmol/L lithium chloride, 1 mL/L Tween 20, 1 mL/L bovine serum albumin, and 5 mL/L Micr-O-protect) was added and the plate was shaken and incubated at room temperature for 30 min. We washed the beads to remove unbound SA-PE and then evaluated them with Bio-Plex[®] 200 system (Bio-Rad, MA, United States). The SA-PE fluorescence measured from each bead was proportional to the number of mRNA transcripts captured by the beads. Expression of target-specific RNA molecules was calculated as the mean values from triplicate cultures and normalized against *Actin* gene (high expression housekeeping gene).

Polymorphism analysis

A standard non-enzymatic method, using the QIA-AMP[®] DNA Blood Maxi Kit (Qiagen GmbH, Hilden, Germany) was used to extract Genomic DNA from peripheral blood leucocytes of all CD patients and healthy controls. In addition, DNA samples from 70 healthy Caucasian subjects (140 alleles) were analysed as controls. Three exon of the *CARD15/NOD2* gene (Exon 4, Exon 8, Exon 11), were amplified by PCR using pairs of primers derived from the published sequence of the gene (available upon request). Each exon is associated with the three main single-nucleotide polymorphisms (SNPs) (R702W-C2104T; G908R-G2722C; 1007fs-3020insC). These three main variants, associated with susceptibility to CD, represented 32%, 18%, and 31%, respectively, of the total CD mutations^[13-15].

The BigDye[®] Terminator Cycle Sequencing kit (Applied Biosystems, CA, United States) was used to perform direct sequencing of PCR amplified products (SNPs rs87950, rs127951, and rs137955) of the *CARD15/NOD2* gene. The samples were analysed in an ABI Prism[®] 310 genetic analyzer (Applied Biosystems, CA, United States). The of the sequences were confirmed with the analysis of newly-amplified fragments and the sequencing of both DNA strands.

Statistical analysis

SPSS software vers. 10 (SPSS Inc., IL, United States) was used to perform the statistical analysis. All comparisons of genes mRNA expression in tissues (non-inflamed and inflamed areas) were performed by non-parametric assay (Mann-Whitney test, Wilcoxon test). Data are reported as mean and ranges unless otherwise stated. A *P*-value < 0.05 was accepted as statistically significant. Furthermore, to better characterize the different clinical CD phenotypes, we compared the results regarding the *CARD15*, *CCR6*, interferon gamma, and *IL-17A* genes to colonic CD patients previously examined for these same genes.

Table 2 Panel of the 24 genes investigated

Symbol	Complete name	Group	Accession number	mim	Gene product function (s)	Ref.
HPRT1	Hypoxanthine phosphoribosyltransferase 1	Low expression housekeeping gene	M26434	308000	It plays a central role in the generation of purine nucleotides, chosen as a low expression housekeeping gene	[39]
ACTB	Actin beta	High expression housekeeping gene	M28424	102630	Is involved in the cell motility, structure, and integrity	[40]
SLC40A1	Solute carrier family 40 (iron-regulated transporter), member 1	1	AF215636	604653	Exports iron from duodenal epithelial cells	[41]
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	1	M14758	171050	Transports various molecules across extra- and intracellular membranes. It belongs to a protein sub-family involved in multidrug resistance	[42]
SLC22A5	Solute carrier family 22 (organic cation/carnitine transporter), member 5	1	AF057164	603377	Transports several small organic cations in the liver, kidney, intestine. It is involved in elimination of drugs and environmental toxins	[43]
SLC22A4	Solute carrier family 22 (organic cation/ergothioneine transporter), member 4	1	AB007448	604190	Polyspecific transporter of organic cations in the liver, kidney, intestine, and involved in the elimination of these molecules.	[44]
CCR6	Chemokine (C-C motif) receptor 6	2	U68030	601835	Induces B-lineage maturation and antigen-driven B-cell differentiation	[45]
IL17A	Interleukin 17A	2	U32659	603149	Produced by Th17-type CD4+ cells. Regulates the activities of NF- κ B and mitogen-activated protein kinases	[26]
IL17F	Interleukin 17F	2	AF384857	606496	Produced by Th17-type CD4+ cells. Stimulates the production of other cytokines, including IL6, IL8. It also inhibits angiogenesis by endothelial cells.	[46]
STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)	2-4	BC014482	102582	Activates transcription of cell growth and apoptosis' genes as responses to inflammation	[22,47]

MICA	MHC class I polypeptide-related sequence A	2	L14848	600169	Acts as a stress-induced antigen broadly recognized by intestinal intra-epithelial gamma delta T cells.	[48,49]
MYD88	Myeloid differentiation primary response gene (88)	2	U84408	602170	Acts as an essential signal transducer in the interleukin-1 and Toll-like receptor signaling pathways	[50]
IL23R	Interleukin 23 receptor	2	AF461422	607562	Expressed on Th17 cells. Involved in the IL23A signaling pathways with the receptor molecule IL12RB1/IL12Rbeta1	[20]
JAK2	Janus kinase 2	2	3717	147796	Is involved in cytokine receptor signaling pathways and is required for responses to gamma interferon	[51]
IFNG	Interferon, gamma	2	3458	147570	It encodes a cytokine with antiviral, immunoregulatory and anti-tumor properties and activates macrophages	[52]
CAMP	Cathelicidin antimicrobial peptide	3	BC055089	600474	It is an antimicrobial protein (defensin)	[53]
CARD15	Nucleotide-binding domain containing 2	2	AF178930	605956	Induces immune response to intracellular bacterial by recognizing the muramyl dipeptide (MDP)	[54]
DEFB4	Defensin, beta 4A	3	AJ314835	602215	Acts as an antibiotic peptide locally regulated by inflammation.	[55,56]
HAMP	Hepcidin antimicrobial peptide	1/2/3	AF309489	606464	It is involved in iron transport, antimicrobial, defence and inflammatory responses	[57,58]
LRRK2	Leucine-rich repeat kinase 2	3/4	AK026776	609007	It is involved in autophagy and implicated in clearance of intracellular bacteria.	[59,60]
TNFSF15	Tumor necrosis factor (ligand) superfamily, member 15	4	AF039390	604052	Induces apoptosis in endothelial cells	[61]
CARD14	Caspase recruitment domain family, member 14	4	AF322642	607211	Regulates the molecular scaffolding process and activates NF-kappa B	[62,63]
ATG16L1	ATG16 autophagy related 16-like 1	4	AK000897	610767	Induces autophagy processes involved in degradation of cell organelles	[64]

ESR1	Estrogen receptor 1	4	X03635	133430	Involved in the metabolic pathway of the hormones and in several diseases including osteoporosis	[65]
BMP2	Bone morphogenetic protein 2	4	650	112261	Induces bone and cartilage formation	[66]
DLG5	Discs, large homolog 5	4	U61843	604090	It encodes for scaffolding molecules involved in cell-cell contact and in the maintenance of epithelial cell integrity. Its products are also involved in the transmission of extracellular signals	[67]

MIM: Mendelian Inheritance in Man. 1: Transport across epithelia; 2: Immune response; 3: Antimicrobial activity; 4: Different physiological activities.

Histological analysis

Once removed, tissue samples were rinsed in 0.1 M, pH 7.0 cacodylate buffer, the same used in prefixation and further steps of histological preparation. Samples were then placed in Karnovsky (1965)^[64], aldehyde solution, and after 3 h prefixation (4°C), underwent prolonged washing in the buffer. Surgery specimens were reduced into approximately 20 mm³ fragments that were post-fixed (1 h 30 min, 4°C) with 1% OsO₄ in cacodylate. These specimens were washed in the buffer, dehydrated in graded ethanol series, soaked in propylene oxide, and embedded in Epon 812. Flat blocks were obtained after polymerization, which were reduced into semi-thin sections (1.5 µm thick), using an 8800 ULTROTOME III LKB equipped with glass knives. Semi-thin sections were stained with borax buffered 1% toluidine blue, and observed with a LEITZ DMRB, in order to collect LM digital images (JPG) for structural analysis. Subsequent ultrastructural observations were carried out on ultrathin sections, obtained with an ULTROTOME NOVA LKB, using a DIATOME diamond knife. Ultrathin sections with gold yellow to silver gray interference colour were selected and collected on uncoated 200-300 mesh copper grids to be electron-dense stained with a hydroalcoholic saturated solution (25 mg/mL) of uranyl acetate, followed by alkaline lead citrate (2 mg/mL). These sections were finally observed (80 KV) with a PHILIPS 201 TEM (BIO, UNIFI), and analogic images were collected, which were later acquired and stored as digital TIFF files using a DIMAGE SCAN DUAL (MINOLTA).

RESULTS

Expression of CD susceptibility genes in the inflamed ileum tissue

The simultaneous expression of 24 genes involved in the pathogenesis of CD was studied in surgical specimens from 14 CD patients with ileal localization of disease. The expression of genes in inflamed ileal mucosa was compared to that of non-inflamed ileal sites collected from the same patient. We observed a significant increase in mRNA levels of twelve genes compared to internal control (Figure 1).

Figure 1 shows that genes related to innate immune response (*NOD 2*, *ATG16L1*, *DEFB4*), and to adaptive immune response (*CCR6*, *IL17A*, *IL17F*, *IL23R*, *IFN-γ*) were significantly increased in inflamed mucosa of CD patients compared with non-inflamed sites. Moreover, the levels of mRNA for genes involved in physiological functions of epithelial cells, such as *JAK2*, *TNFSF15*, and *SLC22A4* were higher in inflamed mucosa compared to non-inflamed mucosa and the differences in expression reached statistical significance.

Detection of *CARD15* polymorphism

DNA samples obtained from peripheral blood were sequenced to investigate the presence of polymorphisms of *CARD15/NOD2* gene. The results of this analysis showed that four patients (28.5%) included in this study are carriers of at least one of the polymorphisms investigated, suggesting that genetic factors might contribute to the dysregulated expression of *CARD15/NOD2* gene^[17-19].

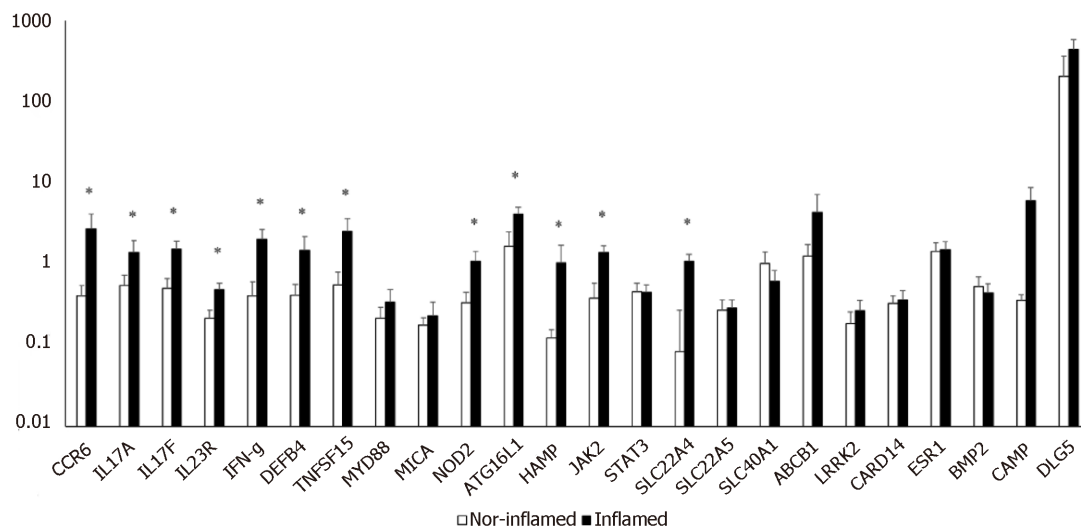


Figure 1 Quantitative evaluation of gene expression using multiplex gene assay in surgical ileum specimens of CD patients. The abscissa shows the genes evaluated in inflamed and not inflamed tissue; the axis of the ordinates shows the value of expression of the gene normalized to the housekeeping actin (gene/ β actin ratio). The first seven genes are more closely related to immunity. P value is reported only when statistically significant ($P < 0.05$).

Morphological analysis

In order to observe the morphology of inflamed tissue samples, light microscopy (LM) and TEM micrographs were obtained. Both gut wall and Lieberkühn crypts retain usual features in both lining epithelium and *lamina propria*. Epithelial cells consist of constitutive enterocytes along with goblet mucocytes, whereas the underlying connective tissue contains large amounts of cells with wide morphological variety (Figure 2). Goblet cells are involved in impressive secretory processes, releasing a moderately opaque product into cryptal and gut lumen through gaps between enterocytes apices (Figure 3). As a consistent pattern, the lamina propria contains granulocytes and plasma cells.

DISCUSSION

Among the numerous genes that have been studied so far with respect to CD, strong and replicated associations have been identified with *NOD2*, *IL23R*, and *ATG16L1* genes^[20]. Environmental factors like smoking, low-fiber and, high-carbohydrate diet, altered GM, and medications such as non-steroidal anti-inflammatory drugs interact with genetic background and induce abnormal inflammation and dysregulation of the immune response. Clinical symptomatology relates to such dysregulation.

The clinical course of CD is conditioned by several parameters such as disease location, extra-intestinal manifestation, and age at onset^[21]. Strictures and fistulas are more frequent in patients with ileal disease, whereas Crohn's colitis remains uncomplicated for many years. On the whole, almost 80% of patients with CD require intestinal surgery, with a permanent stoma required by almost 10%. The presence of selected mutations in the *NOD2* gene (see, e.g., 605956.0001-605956.0003) (IBD1; 266600) has been associated with susceptibility to ileum-localized CD^[22]; patients homozygous for the 1007fs mutation had an early disease onset with long-segment ileal stenoses and entero-enteral fistulas; they frequently needed surgical intervention and had a high risk of recurrence^[23,24]. Beside *NOD2* gene, huge genome-wide linkage-analyses and meta-analyses have described several CD susceptibility regions including IBD5 locus, *DLG5*, and autophagy-related 16-like 1 (*ATG16L1*) gene, *JAK2*, *STAT3* interleukin-23 receptor (*IL23R*), *SLC22A4* and *SLC22A5* *TNFSF15*^[14].

In this paper, we evaluated the expression of 24 genes that were associated to CD susceptibility^[10]. mRNA was extracted from gut specimens obtained from patients with CD ileal localization of CD, undergoing surgery. We used a multiplex gene assay which directly quantifies the mRNA amounts without need of reverse transcription and gives a detailed picture of the inflammation process for each patient^[11]. The same technique was used to quantify gene expression in colonic mucosa from surgical specimens or endoscopic bioptic fragments obtained by CD patients with predominant colonic (L2) location^[10].

The analysis revealed a clear activation of immune-adaptive Th17 response in association with a Th1 response in inflamed mucosa of patients undergoing surgery

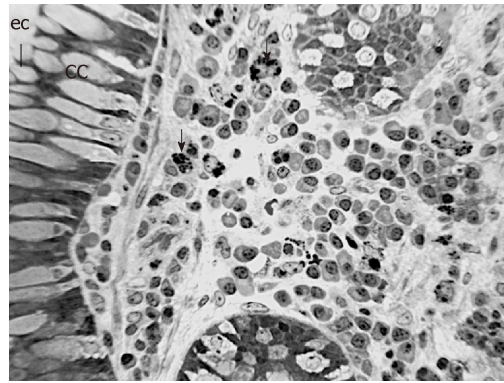


Figure 2 Representative Light microscope (LM) of the lamina propria between Lieberkühn crypts in inflamed ileal tissue of CD patient number 7b. Notice muciparous goblet cells in cryptal epithelium and large amount and variety of immune cells (arrows) in the connective tissue. Semithin (1 μ m thick) section, toluidine blue staining; cc = goblet cell, ec = enterocyte. Scale bar (4 cm) = 70 μ m.

suggesting a dysregulated and very aggressive immune-inflammatory response.

Here gene expression analysis of inflamed ileal mucosa revealed an increased expression of genes involved in adaptive immune response compared to non-inflamed tissue. In particular, we found a significant increase of *IL17A* and *IL17F*, *IL23R* and *CCR6* gene expression suggesting an activation of a Th17 adaptive response^[25,26] similar to that found in gut mucosa of patients with colonic localization. According with this hypothesis, three additional genes involved in Th17 differentiation as *JAK2*, *STAT3* and *TNFSF15*^[27,28] were found to be overexpressed in inflamed ileal mucosa of CD patients compared to non-inflamed sites. Furthermore, as we expected, the expression of the antimicrobial peptides as defensin (*DEFB4*)^[29] and Hepsidin 6 (*HAMP*)^[30] were significantly increased in inflamed mucosa of CD patients compared with non-inflamed sites, suggesting the overwhelming stimulation of epithelial cells by commensal GM. Indeed, while the human β -defensin (*HBD*) 1 is constitutively expressed, other genes, like *HBD2* (gene name *DEFB4*), show pathogen and/or inflammation dependent upregulation^[31] while also being inducible by probiotic bacteria^[32]. Conversely, *HAMP* transcription mediates the effects of host defence and inflammation. Shanmugam *et al.* provided persuasive evidence in support of an important role for the GM composition in influencing hepcidin expression during intestinal inflammation in mouse models of colitis^[33].

As the position of the pathogenic tissue may condition not only the clinical course of the disease but also the probability to require surgery, we also compared with the same methodology (Quantigene 2.0) the expression of selected genes (*IFN- γ* , *CCR6*, *IL17A*, *NOD2*) involved in immune responses in inflamed mucosa with predominant ileal location with the one previously studied^[10] in inflamed mucosa with colonic location. mRNA expression for *IFN- γ* and for the chemokine *CCR6* appeared significantly higher in ileal site compared to colonic site (ileal CD = 2.7 ± 1.5 ; colonic CD = 0.2 ± 0.06 ; $P = 0.01$). The mRNA for *IL-17* and *NOD2* appeared to be expressed at higher levels in ileal site compared to colonic site, even if the difference is not statistically significant ($P \geq 0.05$). The significant differences in the expression levels of *IFN- γ* gene (higher expression in specimens from patients with ileal localization compared to patients with colonic localization) may suggest an increased damage of the ileal mucosa due to the simultaneous presence of Th1 and Th17 effector cells and/or the shift of Th17 cells to Th1 effectors functionally more aggressive than Th17 unshifted cells^[34,35].

Furthermore, according to a worse clinical course of patient with ileal localization of CD compared with patient with colonic localization^[36], the increased expression of *IL17* and *NOD2* in mucosal fragments from patients with ileal CD compared to patients with colonic CD is in agreement with the *NOD2* -dependent regulation of immunity in mouse intestinal tract^[37]. We suppose that the above differences between the two gut tracts (ileal and colonic) may be due to the Paneth cells at the bottom of the crypts of Lieberkühn in the small intestine, which produce antimicrobial peptides and hinder commensal GM and pathogenic bacteria to penetrate gut mucosa. Initially described as innate immune cells producing antimicrobial products, Paneth cells have recently been suggested to constitute a cardinal component of the intestinal stem cell niche. In fact, Paneth cells contribute to controlling the luminal flora as well as repairing the intestinal barrier following an insult. Genomic alterations that impede the Paneth cell compartment functionality can potentially increase the propensity to

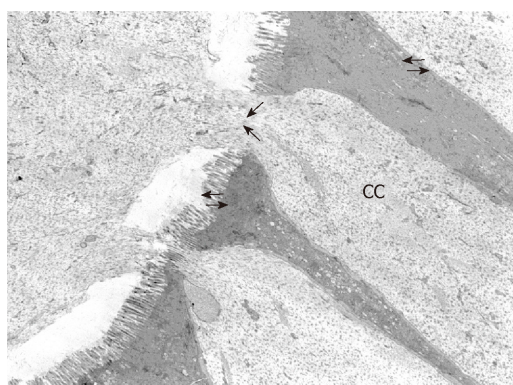


Figure 3 Representative TEM micrograph of Lieberkühn crypt wall and lumen that contains mucous product released (large arrows) by goblet cells (CC) in inflamed ileal tissue CD patient number 7b. Small arrows indicate transport processes involving apical and lateral surfaces of enterocytes. Scale bar (1 cm) = 1 μ m.

develop CD^[38].

As a consistent trait, cryptal goblet cells produce large amounts of mucus that performs the double role of barrier and holder of antimicrobial products. The microscopic anatomy analysis aims to provide some details that illustrate phenotypic features: the large cell variety in the *lamina propria* includes immune lines that represent a further defense tool. Although these morphological traits are not directly related to specific gene outputs, they illustrate the tissue responses to key gene deregulation.

As a pilot study, our study presents a low number of subjects investigated which may have influenced the statistical power of the results. To confirm these results, studies with a larger number of patients are needed. In addition, gene expression was evaluated with Multiplex Gene Assay only. This method directly quantifies the mRNA amounts without need of reverse transcription and gives a detailed picture of the metabolic processes for each patient but it should be validated by comparisons with additional techniques to evaluate gene expression.

One of the main purposes of our research is therefore to identify new molecules involved in metabolic pathways that could potentially represent new biological drugs to identify the appropriate therapy in relation to the clinical phenotype of the CD patient.

ARTICLE HIGHLIGHTS

Research background

The interplay of environmental, genetic and microbial elements influences the etiopathogenesis of Crohn's disease (CD). Differences in the clinical course of CD have recently been reported in patients with ileal or colonic localization of the inflammatory process.

Research motivation

Aim of this study was to define biochemical and histological differences in intestinal biopsies from patients with ileal or colonic localization of Crohn disease in order to identify new assays which can be useful for planning individual therapeutic strategies

Research objectives

Main objective of the current research was to investigate the expression of genes involved in immune-inflammatory pathways in gut mucosa from patients with ileal or colonic localization of CD and to correlate the results of gene expression with those obtained through a classical morphological analysis of surgical biopsies.

Research methods

A Multiplex Gene Assay was used to assess the simultaneous expression of 24 genes related to immune-inflammatory process and to CD pathogenesis. Structural and ultrastructural features of gut samples were also evaluated through Light microscopy (LM) and Transmission Electron Microscopy (TEM) techniques.

Research results

We observed a strong activation of genes involved in TH-1- and TH-17 immune response in patients with ileal localization of CD compared to patients with colonic localization. In addition, the expression of genes for antimicrobial peptides as DEFB4 and HAMP was found highly stimulated in ileal mucosa from CD patients suggesting a possible interference with microbial

commensals at this site.

Research conclusions

Our results indicate that patients with ileal localization of CD have a stronger activation of TH-1 and TH-17 immune-inflammatory responses compared with patients with colonic localization of the disease thus defining a clear subclinical phenotype of CD.

Research perspectives

These results may suggest that therapeutic strategies with biological drugs in CD patients can be differentiated depending on the location of the disease

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