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ORIGINAL ARTICLE

Crohn's Colitis: Development of a multiplex gene expression assay comparing mRNA levels of susceptibility genes

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Summary Crohn's disease (CD) is a multifactorial immunologically mediated disease. In this study we explored, for the first time, the efficacy of the Multiplex Gene Assay technology for detecting mRNA expression profile of 24 selected CD related genes in endoscopic biopsies and surgical specimens from CD patients with colonic localization of the disease. The polymorphisms of genes most frequently associated with CD were also analysed in DNA samples from the same patients. The analysis of endoscopic samples showed increased expression of 7 genes in inflamed mucosa compared to non-inflamed mucosa and suggests the activation of the autophagy process and of a Th17 adaptive response. The analysis of surgical specimens showed increased expression of 16 genes in inflamed tissue compared to non-inflamed internal controls and revealed the activation of immune-adaptive Th17 response in association with a Th1 response.

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Furthermore, an increased expression of genes involved in ionic transport and signal transduction was found in inflamed mucosa compared to non-inflamed internal controls. This study confirms the activation of Th17 and Th1 adaptive-immune response also in colonic CD. It should be stressed that these responses have been disclosed in biopsy tissue, while only Th17 differentiation is revealed in endoscopic tissue. Interestingly, the polymorphisms analysis revealed that a homozygous genotype is associated to a more complicated clinical course.

Introduction

Crohn's disease (CD) is a chronic relapsing condition characterized by a transmural inflammation that can potentially affect any segment of the gastrointestinal tract. It predominantly involves the terminal ileum and colon, and often results in complications such as strictures, perforations and fistulae [1]. The disease affects any age groups, but typically occurs in young adults. Based on predominant characteristics, CD expressions can be assigned to three general phenotypes: stricturing, perforating or inflammatory [2]. Although genetic, immunological and environmental factors are involved in the pathogenesis of CD, aetiology is still unknown and no causal treatment is available. Studies performed on twins and members of the same family confirmed a significant correlation between genetic factors and CD occurrence. Approximately half of monozygotic twins and one third of children of two affected parents develop this disease [3].

International collaborative research, focusing on an unbiased appraisal of the human genome, has been particularly successful in identifying genes and genetic loci that contribute to CD susceptibility [4]. Analyses of the genes and genetic loci involved in CD show several pathways that have a key role in intestinal homeostasis, including barrier function, epithelial antimicrobial defence, innate immune regulation, reactive oxygen species (ROS) generation, autophagy, endoplasmic reticulum stress and main metabolic pathways [5].

Multiplex Gene Assay (QuantiGene Plex 2.0) based on Luminex xMAP Technology is a sandwich branched-chain DNA assay that utilizes cooperative hybridization. The analysis has been chosen because the quantification of mRNA from tissue is direct to specifically capture target mRNA transcripts onto labeled xMAP beads [6]; the resolving power of this technique is large, allowing detection up to 32 genes in simultaneous analysis, thus leading to a single and very detailed phenotypic description for each patient [7].

In this paper, we used Multiplex Gene Assay to analyze differences in gene expression in tissue samples from endoscopies and surgical biopsies of colon mucosa from CD patients. A panel of genes, directly or indirectly related with CD, as suggested by Genome-Wide Association (GWA) studies for Immune Bowel Disease (IBD) and some susceptibility genes shared with different diseases were studied [8].

In particular, we studied:

- the expression of *HAMP, SLC40A1* which are involved in iron metabolism;
- the expression of *IL23R, CCR6, IL17A, IL17F, IFNG, MYD88, TNSF15* which regulate the differentiation of T helper (TH)-1 or TH-17 lymphocytes, *MYD88 CARD15, ATG16L1, CARD14* involved in innate recognition of pathogens and in autophagy;
- *JAK2, STAT3, LRRK2* which modulate cell signalling;
- *BMP2 ESR1* which are involved in bone metabolism and osteoporotic processes;
- *JAK2, HAMP* which are possible target genes for future biological therapies;
- *SLC22A4, SLC22A5* which regulate metabolism of organic cations;
- *ABCB1* which protect the intestinal epithelium against xenobiotics;
- *MICA* which is involved in the interaction with gamma delta T-cells of the intestinal immune system;
- *DEFB4, CAMP, HAMP* defensins which are involved in innate immune response against pathogens;
- *DLG5* which maintains the structure of intestinal epithelium, informing about the status of integrity of epithelial cells.

Materials and methods

Patients

Seven patients affected by colonic CD were recruited at the Gastroenterology and Endoscopy Units of the IRCCS-CSS Hospital at S. Giovanni Rotondo (Italy) (Table 1a). Diagnosis of colonic CD was performed by ileocolonoscopy Seven patients, affected by colonic CD and programmed for surgery, were recruited at the Unit of Surgery, AOUC, University of Florence (Table 1b). For these patients, CD diagnosis was performed by clinical/endoscopic criteria and confirmed by histological analysis. Table 1 shows clinical characteristics of the 14 patients.

Tissue samples from inflamed and non-inflamed colonic areas were obtained from each patient either in endoscopic procedures either in the course of surgery; non-inflamed colonic samples were used as internal controls.

All samples were stored in RNA later (Qiagen, Germany) before homogenization. Peripheral blood was collected in EDTA, and subjected to genomic DNA extraction (QIA amp DNA Blood Maxi Kit, Qiagen GmbH, Hilden, Germany) and genotyping to analyze polymorphisms of relevant genes: the three principal polymorphisms of the gene *CARD15/NOD2* (R702W, G908R, and 1007fs) and some polymorphisms of genes *ATG16L1, IL23R, LRRK2*. We developed a microarray

Table 1 a: clinical characteristics of colonic CD patients undergone endoscopy (endoscopies).

Patient	Pt1a	Pt2a	Pt3a	Pt4a	Pt5a	Pt6a	Pt7a
Localization of CD[a]	L2	L2—L4	L2	L1—L2	L3	L1—L2	L3—L2
Age of CD onset (years)	22	27	55	35	18	42	22
I° Surgery/relapse	I° Surgery	Relapse	I° Surgery	No	I°Surgery	No	No
Disease behavior[b]	B1	B3	B2—B3	B1	B2	B3	B1
Therapy[c]	M, C	M, C	B	B	M, B	M	I
Smoking status[d]	No	Ex-S	No	No	10 cig/day	No	5 cig/day

b: clinical characteristics of colonic CD patients undergone surgery (surgical specimens)

Patient	Pt8b	Pt9b	Pt10b	Pt11b	Pt12b	Pt13b	Pt14b
Localization of CD[a]	L2	L3	L1—L2	L3	L2	L3	L2
Age of CD onset (years)	16	27	24	34	18	28	36
I° Surgery/relapse	I° Surgery	Relapse	I° Surgery	I° Surgery	I° Surgery	I° Surgery	Relapse
Disease behavior[b]	B2	B2, B3	B2, B4	B2	B2, B4	B2, B3	B2
Therapy[c]	B, Ab, C	C, M, I, Ab	I, M	C, B	Ab, M	C	I, Ab
Smoking status[d]	18 cig/day	No	15 cig/day	No	No	Ex-S	No

[a] Localization of CD. L1: terminal ileum, L2: colon, L3: ileum colon, L4: upper G (gastrointestinal).
[b] Disease behavior. B1: nonstricturing/nonpenetrating, B2: stricturing, B3: penetrating, B4: perianal disease.
[c] Therapy. M: mesalazine, I: immunosuppressant, B: biologics, C: corticosteroids, Ab: antibiotics.
[d] Smoking status. Current smoke: no. cigs-day/no. year. Ex-S: ex-smoker.

panel of 24 genes involved in CD pathogenesis and evaluated the co-expression of these genes in samples of inflamed and non-inflamed colon mucosa obtained from endoscopical and surgical specimens of colonic CD patients. Table 2 reports the panel of the investigated genes, the accession number, the number of Mendelian Inheritance in Man (MIM) (used as a reference) and their respective encoded product and function. To facilitate the interpretation of the results, we designed a panel of 26 genes (24 genes which values of expression is normalized by two housekeeping genes) divided in four categories according to their biological function:

- transport across epithelia: SLC40A1, ABCB1, SLC22A5, SLC22A4, HAMP;
- immune response: CCR6, IL-17A, IL-17F, STAT3, MICA, MYD88, IL-23R, JAK2, IFNG, NOD2;
- antimicrobial activity: *CAMP, DEFB4, HAMP, LRRK2*;
- physiological activities: STAT3, LRRK2, TNFSF15, CARD14, ATG16L1, ESR1, BMP2, DLG5.

Multiplex Gene Assay

The mRNA quantization for the genes listed in Table 2 was performed with a Multiplex Gene Assay (Quantigene 2.0, Affymetrix, CA, USA), as previously described [7]. The mRNA expression for *Ccr6, Tnfsf15, Il-17A, Stat3, Card14, Slc40a1, Abcb1, Slc22a5, Atg16l1, Esr1, Il-17F, Bmp2, Card15, Mica, Myd88, Il-23r, Slc22a4, Defb4, Jak2, Dlg5, Lrrk2, Hamp, Camp, Ifn-γ, Actb* (high expression housekeeping gene), *Hptr1* (low expression housekeeping gene), was measured using the QuantiGene multiplex assay (Panomics, Fremont, CA, USA). Samples were lysed after treatment in lysis mixture; mRNA expression in lysates was detected and measured according to manufacturer's instructions. Briefly, samples were frozen in RNA later (Qiagen, Germany). Each sample was weighed and the appropriate lysis solution was added to a final volume of 150 μL containing 50% Lysis Mixture (Panomics) and 1 g/L Mixture (Panomics) and 1 g/L proteinase K. The mixture was shaken at 65 °C for 30 min to lyse the cells. The lysate was stored at −80 °C for later use.

A panel of oligonucleotide capture probes, each with a unique sequence of 15 bases, was covalently linked to carboxylated fluorescently encoded beads (Luminex, Bio-rad, Massachusetts, USA). We mixed each sample lysate diluted at 1:1 and 1:2 with the pooled capture beads in a round-bottom assay well and hybridized for 16 hours at 54 °C (final volume in each well was 100 μL). The assay mixture was transferred to a MultiScreen filter plate (Millipore, Billerica, MA, USA), and unbound material was filter-washed from the wells by rinsing 3 times with wash buffer. The plate was then hybridized at 54 °C for 1 h with 100 μL/well of bDNA amplifier in Amplifier Diluent (Panomics). After the plate was filter-washed twice with wash buffer and incubated at 50 °C for 1 h with 100 μL/well of 5'-dT (Biotin)-conjugated label probe (Panomics) diluted in Label Probe Diluent (Panomics). 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 analysed them with Bio-Plex 200 system (Bio-Rad). 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).

Table 2 Panel of the selected genes investigated in our study.

Symbol	Complete name	Group	Accession number	MIM	Gene product function/s	Reference
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	Fedrigo et al., 2010
ACTB	Actin beta provided	High expression housekeeping gene	M28424	102630	Is involved in the cell motility, structure, and integrity	Vandesompele et al., 2002
SLC40A1	Solute carrier family 40 (iron-regulated transporter), member1	1	AF215636	604653	Exports iron from duodenal epithelial cells	Benyamin et al., 2015
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	1	M14758	171050	Transports various molecules across extra- and intra-cellular membranes. It belongs to a protein sub-family involved in multidrug resistance	Brant et al., 2004
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	Peltekova 2004
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	Girardin et al., 2012
CCR6	Chemokine (C-C motif) receptor6	2	U68030	601835	Induces B-lineage maturation and antigen-driven B-cell differentiation	Fransen et al., 2014
IL17A	Interleukin 17A	2	U32659	603149	Produced by Th17-type CD4+ cells. Regulates the activities of NF-kB and mitogen-activated protein kinases	Pelletier 2010
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	Ueno et al., 2015
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	Noble et al., 2010; Nguyen et al., 2015
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	Allez et al., 2007; Muro et al., 2014
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	Cosmi et al., 2014
IL23R	Interleukin 23 receptor	2	AF461422	607562	Expressed on Th17 cells. Involved in the IL23A signaling pathways with the receptor molecule IL12RB1/IL12Rbeta1	Naser et al., 2012

Table 2 (*Continued*)

Symbol	Complete name	Group	Accession number	MIM	Gene product function/s	Reference
JAK2	Janus kinase 2	2	3717	147796	Is involved in cytokine receptor signaling pathways and is required for responses to gamma interferon	Danese et al., 2016
IFNG	Interferon, gamma	2	3458	147570	It encodes a cytokine with antiviral, immunoregulatory and anti-tumor properties and activates macrophages	Tuller et al., 2013
CAMP	Cathelicidin antimicrobial peptide	3	BC055089	600474	It is an antimicrobial protein (defensin)	Koczulla and Bals, 2003; Koczulla et al., 2003
CARD15	Nucleotide-binding oligomerization domain containing 2	2	AF178930	605956	Induces immune response to intracellular bacterial by recognizing the muramyl dipeptide (MDP)	
DEFB4	Defensin, beta 4A	3	AJ314835	602215	Acts as an antibiotic peptide locally regulated by inflammation	Wehkamp et al., 2009; Gersemann et al., 2008
HAMP	Hepcidin antimicrobial peptide	1/2/3	AF309489	606464	It is involved in iron transport, antimicrobial, defence and inflammatory responses	Verga Falzacappa and Muckenthaler, 2005; Mleczko-Sanecka et al., 2010
LRRK2	Leucine-rich repeat kinase 2	3/4	AK026776	609007	It is involved in autophagy and implicated in clearance of intracellular bacteria	Gardet et al., 2010; Schapansky et al., 2014
TNFSF15	Tumor necrosis factor (ligand) superfamily, member 15	4	AF039390	604052	Induces apoptosis in endothelial cells	Bamias et al., 2003
CARD14	Caspase recruitment domain family, member 14	4	AF322642	607211	Regulates the molecular scaffolding process and activates NF-kappa B	Tsoi et al., 2012; Bertin et al., 2001
ATG16L1	ATG16 autophagy related 16-like 1	4	AK000897	610767	Induces autophagy processes involved in degradation of cell organelles	Parkes, 2012
ESR1	Estrogen receptor 1	4	X03635	133430	Involved in the metabolic pathway of the hormones and in several diseases including osteoporosis	Aguirre et al., 2007
BMP2	Bone morphogenetic protein 2	4	650	112261	Induces bone and cartilage formation	Cejalvo et al., 2007
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	Stoll et al., 2004

MIM: Mendelian inheritance in man; 1: transport across epithelia; 2: immune response; 3: antimicrobial activity; 4: different physiological activities.

Polymorphism analysis

Genomic DNA was extracted from peripheral blood leucocytes of all CD patients and healthy controls by a standard non-enzymatic method, using the QIAamp DNA Blood Maxi Kit (Qiagen GmbH, Hilden, Germany). In addition, DNA samples from 70 healthy Caucasian subjects (140 alleles) were analysed as controls. Three coding exons of the *CARD15/NOD2* gene, of the associated with the three main single-nucleotide polymorphisms (SNPs) are as follows:

- exon 4, R702 W (C2104T);
- exon 8, G908R (G2722C);
- exon 11, 1007fs (3020insC).

These coding exons were amplified by PCR using pairs of primers derived from the published sequence of the gene (available upon request). These three main variants associated with susceptibility to CD, represented 32, 18, and 31%, respectively, of the total CD mutations.

Direct sequencing of PCR amplified products (SNPs rs87950, rs127951 and rs137955) of the *CARD15/NOD2* gene was performed using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). The samples were analysed in an ABI Prism 310 (Applied Biosystems) Genetic Analyzer and sequence variations were confirmed analysing newly amplified fragments and sequencing both DNA strands. Samples were genotyped for the SNPs of genes *ATG16L1, LRRK2, IL-23R* (in particular we considered the rs7517847 and rs11209026 for *IL-23R* rs2241880 for *ATG16L1*, rs11564258 and rs3761863 for LRRK2) using 5′exonuclease TaqMan genotyping assays on an ABI Prism 7900 Real-Time polymerase chain reaction (PCR) System, according to manufacturer's instructions (Applied Biosystems, Foster City, CA).

Statistical analysis

Statistical analysis was performed using SSPS software (SPSS, Inc, Evanston, IL). All comparisons of genes mRNA quantization in tissues (inflamed and non-inflamed areas) were performed by Wilcoxon test. Data are reported as mean and ranges unless otherwise stated. A P value of < 0.05 was accepted as statistically significant.

Results

Expression of CD susceptibility genes in the inflamed colon

The simultaneous expression of 24 genes involved in the CD pathogenesis was studied in colonic endoscopic biopsies ($n = 7$) and surgical specimens ($n = 7$) from patients with Crohn's colitis. Gene expression in inflamed colonic mucosa was compared to that of non-inflamed mucosa collected from the same patient.

Gene expression in endoscopic samples

The analysis of data obtained from endoscopic samples revealed a significant increase in mRNA level of 7/24 genes compared to control tissues (Fig. 1, panel A). In particular significant differences between inflamed and non-inflamed mucosa were found in the expression of molecules and receptors related to TH17 adaptive response as interleukin 17 A (*Il-17A*, $P = 0.018$), Interleukin 17 F (*Il-17F*, $P = 0.028$), Interleukin 23 Receptor (*Il-23r*, $P = 0.043$) and defensin beta 4 (*Defb4*) ($P = 0.018$). Significant differences were also found in the expression of Autophagy Related 16-like1 (*Atg16l1*, $P = 0.028$) Janus Kinase 2; (*Jak2*, $P = 0.028$) and Leucine-Rich Repeat Kinase 2 (*Lrrk2* $P = 0.018$). The level of mRNA for the Defensin Beta4 (*Defb4*) showed the greatest absolute value of expression, both in inflamed and non-inflamed areas.

Gene expression analysis in surgical specimens

The gene expression analysis in surgical specimens obtained from patients with Crohn's colitis revealed an increased expression of 15/24 genes in inflamed tissues compared to non-inflamed tissues. (Fig. 1, panel B). In particular the compared analysis of gene expression in inflamed and non inflamed mucosa revealed higher expression of genes involved in TH17 response as *Ccr6* ($P = 0.018$), *Il-17A* ($P = 0.018$), *Il-17F* ($P = 0.018$), *Il-23r* ($P = 0.018$) as well as in autophagy *Atg16l1* ($P = 0.018$), thus confirming the results obtained with endoscopic samples. Beside genes involved in TH17 response, we also found higher expression of *Ifnγ* ($P = 0.018$) suggesting the simultaneous activation of TH1 response and/or the presence of IFN-γ producing cells of innate immunity. According with the high inflammatory status of colonic mucosa from patients subjected to surgery we found increased expression of genes involved in iron transport/antimicrobial defense *Hamp* ($P = 0.028$), iron export from duodenal epithelial cells *Slc40a1*, ($P = 0.043$), signal transduction *Stat3*, ($P = 0.018$) in cytokine receptor signaling pathways *Jak2* ($P = 0.043$), multidrug resistance *Abcb1*, ($P = 0.018$), in elimination of drugs and environmental toxins *Slc22a5* ($P = 0.018$) and finally in cell—cell contact and in the maintenance of epithelial cell integrity *Dlg5* ($P = 0.018$).

In addition, the products of these 15 genes are also involved in the transmission of extracellular signals *Dlg5* ($P = 0.018$).

The remaining genes (*SLC22A4, MYD88, MICA, CAMP, TNFSF15, CARD14, ESR1*) exhibited no significant differences of mRNA levels, but their values might be taken into account to assess which metabolic pathway is active at that time of the investigation.

Detection CARD15, ATG16L1, LRRK2, IL-23R polymorphism

To investigate the influence of genetic factors [7] on the quantitative expression of the immune-related genes, we sequenced DNA samples obtained from peripheral blood of all patients for the presence of polymorphic genes, frequently associated to CD as *CARD15* [9], *ATG16L1* [10], *LRRK2* [11,12], *IL-23R* [13,14].

All CD patients included in this study, either donors of endoscopic biopsies or of surgical specimens, showed of at least one polymorphism in heterozygosis, suggesting the involvement of genetic factors in the dysregulated expression of the reported genes. Table 3 shows the complete list

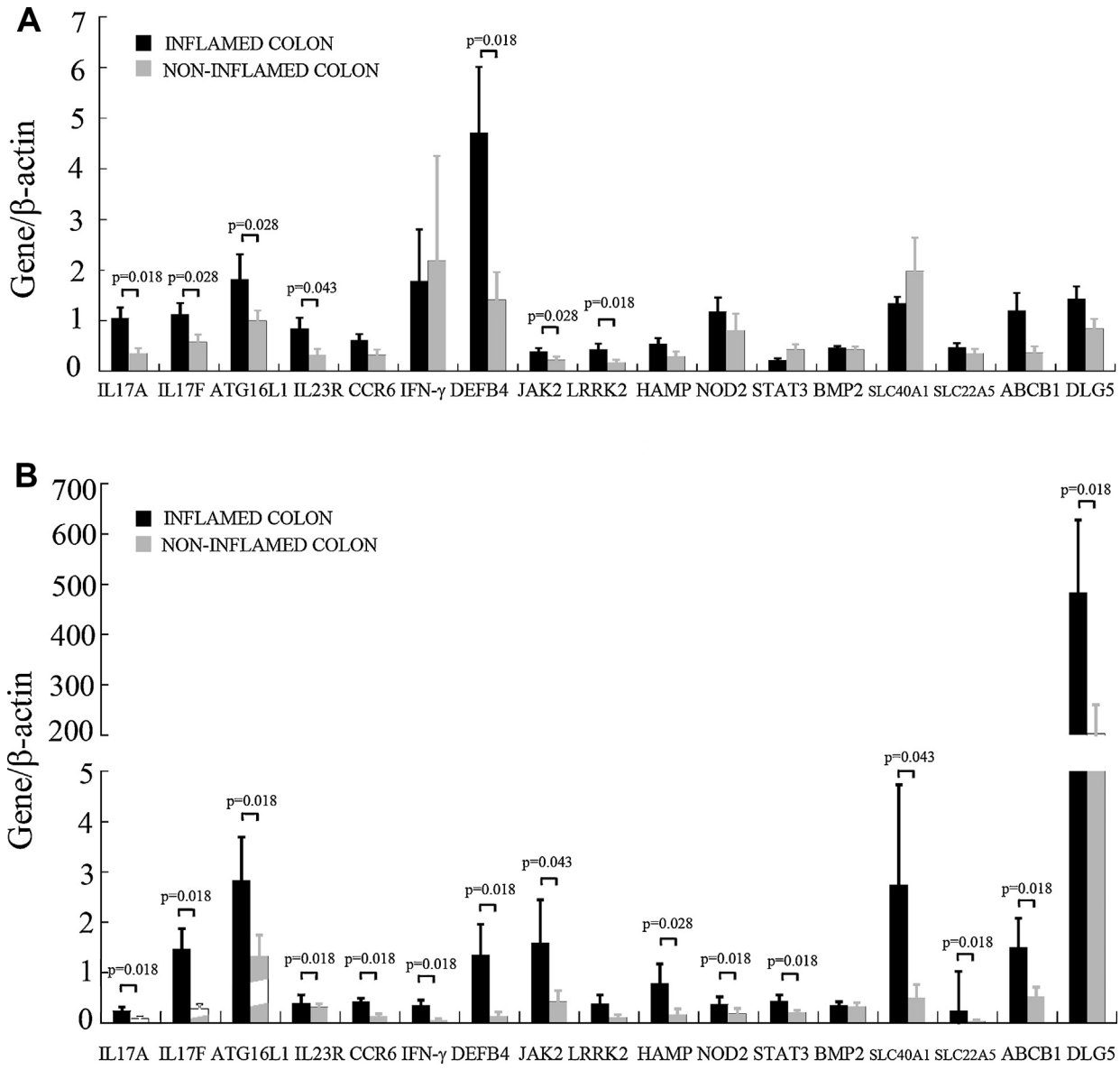

Figure 1 Quantitative evaluation of gene expression using Multiplex gene Assay (Quantigene 2.0) in endoscopic tissues (Panel A) and surgical specimens (Panel B) of CD patients. The abscissas shows mRNA levels of the genes compared to controls; the axis of the ordinates shows the value of expression of the gene normalized to the housekeeping actin (gene / β actin ratio).The first seven genes of both panels (A and B) are more closely related to immunity. P value is reported only when statistically significant ($P < 0.05$).

of polymorphisms found in our CD cohort as well as the details of the analysed SNPs. Interestingly, matching clinical features of patients (Table 1) with SNPs status (Table 3), we observed that patients with a homozygous and double-heterozygous genotype underwent to a more complicated clinical course of CD, since they were affected with a surgical recurrence of CD.

Discussion

Current hypothesis for CD pathogenesis emphasizes the loss of immunological tolerance and the altered permeability of epithelial mucosa as responsible for an abnormal inflammatory immune response against antigens commonly found in the intestinal lumen. In this way, a chronic inflammatory process is established [15]. These observations, combined with the presence of family history for the disease [16], leads to assume that the pathogenesis of CD is the result of the interaction of at least three factors:

- genetic susceptibility [17];
- immunological priming by the enteric microflora [18];
- tissue damage mediated by the immune system [19].

The loss of tolerance and the consequent adaptive-immune response are amplified in the presence of defects in mechanisms of early removal of invading bacteria. In particular, dysfunction of autophagy caused by genetic/phenotypic variations within CD susceptibility genes, such as *ATG16L1* and *NOD2/CARD15* functions have

Table 3 List of SNPs in patients undergone endoscopy (a) and surgery (b).

a. Endoscopic tissue

	SNP	Pt1a	Pt2a	Pt3a	Pt4a	Pt5a	Pt6a	Pt7a
CARD15	rs 87950	wt	wt	wt	wt	wt	wt	wt
	rs 127951	wt	wt	wt	wt	hz	wt	wt
	rs 137955	wt	wt	wt	wt	hz	wt	wt
IL23R	rs 7517847	wt	hz	wt	hz	wt	wt	wt
	rs 11209026	wt	hz	wt	wt	wt	wt	wt
ATG16L1	rs 2241880	wt	wt	hz	wt	hz	hz	hz
LRRK2	rs 11564258	wt	hz	wt	wt	wt	wt	wt
	rs 3761863	hz	homo	hz	wt	hz	wt	hz

b. Surgical specimen

	SNP	Pt8b	Pt9b	Pt10b	Pt11b	Pt12b	Pt13b	Pt14b
CARD15	rs 87950	wt	wt	wt	wt	wt	wt	wt
	rs 127951	hz	homo	wt	wt	wt	wt	wt
	rs 137955	hz	wt	wt	wt	wt	wt	wt
IL23R	rs 7517847	wt	wt	wt	hz	wt	wt	hz
	rs 11209026	wt	wt	wt	wt	wt	wt	wt
ATG16L1	rs 2241880	hz	homo	hz	wt	hz	hz	wt
LRRK2	rs 11564258	wt	wt	wt	wt	wt	hz	wt
	rs 3761863	hz	hz	wt	wt	hz	hz	hz

wt: wild type; hz: heterozygous; homo: homozygous; Pt: patient.

been involved [20,21]. Genome-Wide Associations studies [22] are very useful for the identification of susceptibility genes; however, only studies of co-expression which potentially capture the inflammatory process in its complexity, can show homeostasis alteration relevant in the CD pathogenesis.

In this study, we have investigated through Multiplex Gene Assay the expression profile of selected genes involved in immune-inflammatory response in endoscopic biopsies and surgical specimens from patients with colonic CD. In order to achieve a complete assessment of immune homeostasis in inflamed tissue, we collected also non-inflamed colonic mucosa samples for each patient. (Fig. 1A, B) The use of Multiplex Gene Assay allowed us to obtain a detailed analysis of immune-inflammatory events arising in the course of the disease. In particular, the involvement of a TH-17 response was strongly suggested by the dysregulated expression of *CCR6, IL-17A, IL-17F,* IL-23R found in all samples either from endoscopy either from surgery. The involvement of TH-1 mediated cellular response is suggested by the higher expression of *INF-*γ in inflamed mucosa from patients subjected to surgery (Fig. 1B).

The nature of T helper cell development in CD has been an area of discussion for several years. The predominating hypothesis has been that the adaptive-immune response in CD is dominated by Th1 cells [23]. However in the last years, the identification of Th17 as a unique subset of T helper cells has challenged this view, and today some authors argue that the adaptive-immune response in CD is dominated by Th1 and Th17 cells [24].

The use of Multiplex Gene Assay allow us to detect significant higher expression of *Il-23r, Il17-A*, and *Il-17F* genes compared to the respective controls (not inflamed tissue), strongly supporting the involvement of Th17-mediated adaptive response in the pathogenesis of Crohn disease [25]. As Th17 cells produce *Il17A* and *Il17F*, whose genes are co-expressed on the same chromosomal region [26,27], the presence of Th17 in CD gut tissues examined is suggested not only by expression of both *Il-17A, Il-17F* mRNA, but also by the expression of *Il-23r* mRNA, a membrane receptor expressed by CD4+ cells which drives Th17 differentiation [28,29].

As further proof of a TH17-mediated response, the Defensin beta 4 gene was strongly increased compared to non-inflamed tissues in both type of samples. Defensin beta 4 is a typical antimicrobial peptide, whose production by epithelial cells is strongly induced by Th17 effector T cells through the combined production of Il-17 and Il-22. Colonic *Hbd2* was dysregulated at mRNA and protein level in CD [38]. A higher expression of the Hepcidin gene (*Hamp*) and of solute carrier family 40 member 1 (*Slc40a1)* gene was also found in inflamed mucosa compared to non-inflamed tissues but it was limited to surgical samples. These genes code for antimicrobial peptides typically produced in the context of a Th17 response [30] strongly involved in regulating iron availability at mucosal levels and in preventing pathogen colonization [31]. The high expression of molecules involved in iron transport as hepcidin and *Slc40a1* might be responsible for the down-regulation of iron cell release, leading to hyposideremia (the so-called ''anaemia by inflammation'') [32] so that the use of drugs anti-hepcidin could be helpful in the treatment of *anemia* in patients with chronic inflammation. In this regard, it has been determined that hepcidin binding to ferroportin leads to the binding and activation of the protein Janus Kinase2 [33], therefore also in this way *JAK2* appears to be a possible target for CD pharmacological treatment [34]. Numerous clinical trials have recently demonstrated that inhibitors of *JAK2* are

safe and efficacious, as they inhibit the entire metabolic pathway [35].

Despite the numerous evidences for the role of Th17 cells, these cells are unexpectedly rare in the tissues and biological samples of patients with chronic inflammatory disorders [36]. One possible explanation may be that Th17 cells show a transient phenotype, resulting from their tendency to shift into Th1 cells in the context of inflammatory microenvironments. Th17 cells rapidly shift into the Th1 phenotype in the presence of IL-12 and/or TNF-α as well as by the fact that they possess self-regulatory mechanisms limiting their own expansion [37,38]. In our case, it is a great expression of the Ccr6 gene, a surface determinant expressed by TH17 lymphocytes [39] was found only in inflamed mucosa from surgical specimens of CD patients. In the same specimens, we also evidenced high expression of Ifn- γ gene, a finding which may suggest the differentiation of TH17 lymphocytes [30] to TH1 cells, that underscores the progression toward a more aggressive and more pathogenic phenotype compared to the TH17 unshifted cells [37]. These Th17-derived Th1 cells, named non-classic Th1 cells, were proposed as a possible target for the therapy of inflammatory disorders.

The differences that we have highlighted between the gene expression in biopsies from endoscopies and intraoperative tissue, reveal that the answer to the insult by pathogens is divided into a succession of phases that are represented by elements of the innate response in endoscopic tissue of the colon and the acquired immunity response in the deeper tissues such as intraoperative biopsies.

From this study, we expect that these genetic insights will transform the landscape of common-complex diseases; we might characterize them better for therapeutic purposes if we can ''isolate'' the set of genes specific for each disease [8].

Conclusions

In conclusion, the use of advanced Multiplex Gene Assay technologies allowed to confirm the relevance of the immune-inflammatory response mediated by Th17 related molecules in the pathogenesis of colonic CD. Data obtained in surgical specimens also suggest that TH-17 cells may progress toward a more aggressive TH-1 phenotype in advanced stages of the disease. Overall, our findings provide a further rationale for the use of antibody treatment against Il-23 with anti-Il-12Rbeta1 and anti-Il-23R antibodies in CD patients [40,41].

Ethics approval and consent to participate

This study was approved by EC of AOUC of Florence, on May 2nd 2011, no. protocol 2011/0016888, rif. 95/10, authorization Gen Dir 17/572011 no. protocol 2011/0018055 and written informed consent was obtained from all study subjects.

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Disclosure of interest

The authors declare that they have no competing interest.

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