Inflammatory bowel disease classification through multigene analysis: fact or fiction?

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"Although the etiology of ulcerative colitis and Crohn's disease are closely related, both conditions are immunologically distinct diseases with clear differences in their cytokine expression profiles."

Inflammatory bowel disease (IBD) is a term that encompasses several intestinal conditions of chronic inflammation in the gastrointestinal (GI) tract. The pathogenesis of IBD is a complex process, involving environmental, genetic, microbial and immune factors. Currently, it is assumed that all components are necessary to have the typical manifestations of IBD, but, in reality, it is unclear to what extent each factor contributes to the disease process, and whether some are more important than others. However, one essential step seems to be the increased permeability of the epithelial barrier, which also contributes to the pathogenesis of several other GI disorders, such as celiac disease, food allergy and irritable bowel syndrome (IBS) [1,2].

Irritable bowel syndrome can be classified into different entities with common symptoms and features, although they sometimes overlap. In both major entities of IBD, ulcerative colitis (UC) and Crohn's disease (CD), the immunological balance at the intestinal barrier is severely impaired and shifted towards the proinflammatory side, with the expression of cytokines causing chronic mucosal inflammation specific to UC or CD. Ideally, these specific immunological characteristics should be reflected in gene expression signatures specific to the disease.

Diagnostic challenges in inflammatory bowel disease

Although the etiologies of UC and CD are closely related, both conditions are immunologically distinct diseases, with clear differences in their cytokine expression profiles. While CD is associated with an inflammatory process of Th1 and Th17 immune response reflected by increased secretion of IL-12, TNF-α, IFN-γ and IL-17, UC is associated with an atypical Th2 immune response reflected by increased secretion of IL-5 and IL-13 leading to the cytolysis of epithelial cells [3–5].

Despite these differences in the two diseases, they can appear phenotypically quite similar. UC and CD share features and neither disease has a pathognomonic finding present in every case of one and absent in every case of the other disease, which can make the differential diagnosis a challenging process.

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Different retrospective studies analyzing the reclassification from UC to CD or *vice versa* showed that between 3 and 9% of patients were reclassified. So far, there are no standard biomarkers for IBD and the diagnosis is typically based on clinical symptoms, patient history and endoscopic and histopathological findings but often the full structural changes detectable by endoscopy or histopathology develop over a substantial period of time. Therefore, the diagnosis may be delayed for 6–12 months [6]. Overall, the

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diagnostic accuracy of pathologists in classifying the IBD type in multiple biopsies is 60–74%, with uncertainty occurring not only in patients seen at the earliest stages of disease, but also in patients presenting with fulminant colitis and in those having already received medical treatment, as various treatments can change the typical disease pattern [7,8].

Despite the recent advances in medical therapy, surgery is required in 30–40% of patients with UC and approximately 25% of patients with colonic CD (up to 80% if the small intestine is involved). For patients with UC the surgery may be curative, whereas a recurrence of CD following surgery is common. A reliable differential diagnosis between UC and CD is particularly important in patients for whom a colectomy and construction of an ileal pouch anal anastomosis (IPAA) is considered, as this type of restorative surgery is generally not appropriate for CD.

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In UC the risk of colonic cancer is elevated and dependent on the duration and extent of the colitis; therefore, a regular colonoscopic surveillance is widely recommended.

In 10–30% of cases presenting with IBD, no firm diagnosis can be made after endoscopic and histological evaluation, and a temporary diagnosis of 'IBD unclassified' (IBDU) is assigned [9]. Clinical practice has taught us that a vast proportion (up to 80%) of patients are eventually reclassified as being affected by either CD or UC, and up to 20% as not affected by IBD [10]. However, reassessment of diagnosis may take up to 10 years, sometimes even longer. Complicating the issue even further, is that a small subset of patients exists in which no firm diagnosis can be made, even after resection. These cases are classified as indeterminate colitis or as 'colitis of uncertain type and etiology' [8], a condition that some believe may represent another separate clinical entity. If this proves to be the case, the need for a reliable test to provide a more robust diagnosis of IBDU is even more evident and it would enable the possibility for better disease management for the clinician and patient.

Genomic analysis & IBD classification

Genomic analyses aim to identify sequence differences and mutations in either copy of a gene that increase the risk of being afflicted with a disease. Genomic analysis has contributed greatly to our understanding of the pathogenesis of IBD and has further demonstrated that genetic predisposition is an important factor in IBD etiology. Genetic studies have also implicated that genes which control the innate immune response are of great importance in the development of IBD and further emphasized the necessity of a balanced interaction with the intestinal microflora, leading to the concept of a defect in the intestinal barrier as being a key defect in the pathogenesis of CD [11]. In particular through the genome-wide association studies, it has so far been possible to identify over 30 IBD-linked genetic variations, such as the *IL23R* polymorphisms which revealed that the IL-23 pathway is important in IBD pathogenesis.

The challenge of genetic analyses lies in the heterogeneity in the diseases and that some genetic aberrations impart a higher propensity to develop one form of IBD than the other. For example, the genetic contribution of the susceptibility gene *NOD2/CARD15* as well as *IBD5* locus genes is evident in CD, whereas a mutation in the *HLA-DRB1*0103* allele has a strong impact on the severity of UC. In populations from Europe and North America *NOD2/CARD15* determinants are believed to be important factors in the etiology of CD but not in the Japanese or Korean populations. More intriguingly, there are even differences within European populations. In Northern countries, such as Sweden, Norway, Iceland, Scotland and Ireland, the carriership of mutations in the *CARD15* gene is much less frequent, despite the fact that both Scandinavia and Scotland represent high-incidence countries. Equally, the disease severity is higher in these countries [12,13]. Although the number of known susceptibility genes and their genetic variations is rising, they are only explaining fewer than 30% of IBD cases, which shows that genetic analysis is not (yet) sufficient to classify IBD [14,15].

Gene expression & IBD classification

Just as genome-wide scans and candidate gene analysis have greatly enhanced our efforts to define the pathogenic mechanism of IBD, gene expression technologies have been employed to identify genes whose expression status has changed within the diseased mucosa. Yet few studies have applied mRNA profiling to complex disease tissue like the intestinal mucosa, reflecting the unique challenges inherent to this type of analysis. The identification of global differences in protein expression from biological samples imposes an even greater challenge, and would be highly desirable. Despite the recognition of better methods of protein isolation and identification, a number of proteins or serological factors do exist that are currently in clinical use. They include serological markers (antimicrobial antibodies) and stool markers and have the common advantage of being noninvasive, which brings us to the common disadvantage of not being specific for IBD.

The serological markers p-ANCA, ASCA and the proprietary markers anti-OmpC and anti-Cbir are useful additions to complement histological examinations, but are not sufficient to base an initial diagnosis on [16]. For example, up to 50% of patients at onset of the disease can be serologically negative [17].

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Despite these shortcomings, it seems that disease progression (severity and need for surgery) correlates with the presence and level of antimicrobial antibodies, such as ASCA, anti-OmpC and anti I2 [18,19]. The presence of multiple antibodies

is accompanied by a more accelerated progression of CD. It is also useful to note the correlation of high p-ANCA levels in UC patients after IPAA with chronic pouchitis or in p-ANCA-positive CD patients (~20% of CD patients), who seem to have a less severe disease progression.

Fecal markers, such as calprotectin and lactoferrin, are sensitive indicators of inflammation but are not specific for IBD (diverticular disease, infectious enterocolitis and cancer). It is proposed that they are useful in pediatric patients to establish whether invasive testing is required [20] and are very efficient in detecting pouch inflammation following IPAA (IBD11 [21,22]). It also seems that elevated levels of calprotectin after remission can predict a relapse in UC and to a somewhat lower extent also in CD patients [23].

These biological markers offer an important addition to the clinical assessment in form of disease progression and some of these markers, or indeed others, may prove to be of real value, but endoscopic and histological evaluations still remain the tools of choice to diagnose and classify an IBD patient [16].

In the late 1990s, when cDNA- and oligonucleotide-based microarrays were sufficiently developed to enable the measurement of mRNA expression levels of hundreds of genes simultaneously, the technology became widely used addressing a whole variety of diseases including IBD. The objectives were relatively straightforward: to find genes involved in the pathogenesis of IBD that could equally serve as potential biomarkers for the differential diagnosis of UC and CD.

First, such studies utilized resected colonic specimens, which resulted in the identification of gene families and functional groups of genes that are dysregulated in IBD and probably involved in the pathogenesis as, for example, detoxification enzymes, ion transport mediators, antimicrobial genes and inflammation mediators [24,25]. Gene expression analysis in cell cultures gave insight into the effect of treatment of for example 5-aminosalicylic acid on $CaCo_2$ cells [26]. However, this was rapidly superseded by the use of endoscopic biopsy tissues to yield expression patterns more closely related to the disease allowing disease subtypes to be categorized [27,28].

Despite the obvious fact that such microarray-based approaches offer a high-throughput possibility, the use of such technologies came with a price insofar as they query a large number of genes, many of which are ultimately not relevant to the specific disease process. This inevitably makes rational interpretation of the huge quantity of data generated a challenging task.

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Perhaps a more manageable approach is offered by methods that, through a form of molecular subtraction termed subtractive suppression hybridization [29], enrich genes on the basis of their abundance and thereby generate cDNA libraries that contain a high proportion of dysregulated genes that are specific to the diseases in question. This approach was successfully applied to cases of IBD whereby resected biopsy specimens from the inflamed colon of IBD patients were enriched for dysregulated genes. The enriched expression profiles were then screened against cases of UC and CD, which led to the identification of seven potential biomarkers that through further evaluation proved to be very effective at discriminating UC from CD and equally IBD from IBS [30]. These biomarkers were identified as solute carrier 6A14 (*SLC6A14*), *SLC26A2*, small protein associated with PDZ domain-containing protein (*SPAP*)-1, regenerating protein IV (*RegIV*), Vanin-1, matrix metalloproteinase (*MMP-7*), and growth-related oncogene-α (*GRO*-α). These genes are reported to be involved in biological processes as transmembrane transport, inflammation, tissue repair and extracellular matrix turn-over, as well as carcinogenesis. By analyzing the expression profiles of these genes in different patient biopsy samples by quantitative PCR, they could demonstrate that UC, CD and IBS have distinct expression signatures.

By constructing a special algorithm based on the quantitative and qualitative differences of the biomarkers (relations of the biomarkers to each other), it was possible to convincingly predict the probability of a patient having one of these diseases: UC, CD or indeed none of them, with high sensitivity and specificity values. More importantly, the multigene analysis of IBDU cases illustrated the capability of specifying a diagnosis in the absence of any specific histopathological features.

The method described by von Stein and colleagues illustrates the feasibility of adopting a mRNA expression profiling approach to isolate and identify specific biomarkers, whose collective dysregulation forms the basis of a sensitive discriminator that successfully segregates IBD subtypes.

The acknowledgement that UC and CD are not only two disease entities, but rather two disease entities having distinct expression signatures, opens up the possibility to address also other GI disorders such as diverticular disease and microscopic colitis. Indeed, a first glance at the expression signatures of a few diverticulitis and collagen colitis cases showed again differences in the expression signature of these genes.

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However, the real challenge lies in whether the ability to correctly determine IBD subtypes in the absence of distinguishing clinical features allows the implementation of more appropriate treatment regimes for the patient.

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