Molecular Biomarkers for Inflammatory Bowel Disease

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Diagnosis and prognosis of inflammatory bowel disease (IBD)—a chronic inflammation that affects the gastrointestinal tract of patients—are challenging, as most clinical symptoms are not specific to IBD, and are often seen in other inflammatory diseases, such as intestinal infections, drug-induced colitis, and monogenic diseases. Laboratory testing of blood or feces has the advantage of being non-invasive, rapid, cost-effective, and standardizable. Although the specificity and accuracy of laboratory testing alone need to be improved, it is increasingly used to monitor disease activity or to diagnose suspected IBD cases in combination with endoscopy and/or imaging.

IBD biomarkers

1. Introduction

Inflammatory bowel disease (IBD) is a set of chronic and idiopathic inflammatory conditions that affect more than 3.5 million patients worldwide. The two major forms of IBD are Crohn's disease (CD), in which inflammation affects any segment of the gastrointestinal (GI) tract [1], and ulcerative colitis (UC), in which inflammation affects the inner lining of the colon or rectum [2]. Patients with IBD are up to six times more likely to develop colorectal cancer than the general population [3][4]. In addition to the molecular alterations (such as chromosomal instability, microsatellite instability, and hypermethylation) that contribute to sporadic colorectal cancer, IBD-related colorectal cancer is linked to inflammation that induces the transcription of mutated cancer genes [5]. Loss-of-function mutations in tumor-suppressor protein p53 occur in both sporadic and IBD-related colorectal cancer, but they occur earlier in the non-dysplastic mucosa of IBD-related colorectal cancer than in sporadic colorectal cancer [4][5]. Another mutation observed in both types of cancer is the nonfunctional adenomatous polyposis coli (APC) gatekeeper gene. Unlike the p53 mutation, APC mutation occurs just prior to carcinoma in IBD-related colorectal cancer, but at a much earlier stage in sporadic colorectal cancer [4]. Other gene mutations linked to IBD-related colorectal cancer include p27, k-Ras (12p12) oncogene, human mismatch repair genes (e.g., hMLH1, hMSH2), and p16 [4].

CD and UC are both characterized by mucosal inflammation, with occasional flares and remittance. Inflammation in CD can affect any segment of the GI tract, and spreads in a non-continuous pattern [1][6]. CD commonly involves the formation of strictures, abscesses, and fistulas [6]. Its histological features include thickened submucosa, fissuring ulceration, transmural inflammation, and non-caseating granulomas [6]. Inflammation in UC affects the inner lining of the colon or rectum, and spreads in a continuous pattern [2][6]. It shows superficial inflammatory changes in the mucosa and submucosa, and involves the formation of cryptitis and crypt abscesses [6]. The clinical

symptoms of IBD include abdominal pain, diarrhea, rectal bleeding, weight loss, nausea, intestinal pain and, in some cases, fever [7][8]. As these symptoms are not specific to IBD, the clinical diagnostic process must consist of using a combination of endoscopic, radiological, clinical, histological, and laboratory tests [9]; a single technique is often insufficient for the diagnosis.

Endoscopy and imaging are essential techniques for the diagnosis, management, and treatment of IBD. They are used in the initial evaluation of patients with suspected IBD, as well as in making a differential diagnosis of UC versus CD in confirmed IBD cases [10]. The strength of endoscopy as a diagnostic tool lies primarily in its ability to visually observe different bowel segments, allowing clinicians to assess disease severity and monitor disease activity over time. Ileocolonoscopy has traditionally been the most used form of endoscopy in IBD. The initial evaluation of patients presenting with clinical symptoms suggestive of IBD should be carried out with ileocolonoscopy, as recommended by the American Society for Gastrointestinal Endoscopy (ASGE) Standards of Practice Committee [11]. In addition to providing a visual of the colon and the terminal ileum, ileocolonoscopy can be used to obtain biopsy specimens for further analysis. The ASGE suggests obtaining at least two biopsy specimens from five sites throughout the bowel during the initial evaluation [12]. However, the invasiveness and high cost of ileocolonoscopy are major drawbacks that have limited its frequent use for monitoring disease activity.

New, less-invasive endoscopic techniques that can more accurately diagnose IBD, while also providing a differential diagnosis of CD and UC, have emerged in the past few years. These include video capsule endoscopy (VCE), confocal laser endomicroscopy (CLE), and single- or double-balloon-assisted enteroscopy (SBE and DBE, respectively). VCE provides imaging of the whole bowel via ingestion of a wireless capsule endoscope [13]. This technique is particularly useful for inspecting areas in the GI tract that cannot be visualized by colonoscopy [14]. Although the risk of capsule retention is low, it remains the primary concern in patients with suspected or known IBD [15]. VCE is less invasive and more cost-effective than ileocolonoscopy, but it cannot be used in performing biopsies. In CLE, a confocal laser microscope is used in vivo to obtain living tissue images during colonoscopy [16]. CLE has the advantage of offering a faster diagnosis than a traditional colonoscopy. Enteroscopy in both of its forms (SBE and DBE) allows access to small bowel areas that standard endoscopy cannot reach. Additionally, enteroscopy can be used in performing histological analysis. However, due to its technical complexity and time-consuming preparation, enteroscopy is not recommended for the initial evaluation of suspected IBD cases [17].

In confirmed IBD cases, clinical symptoms alone are insufficient for clinicians to determine the extent of mucosal inflammation, or to make a differential diagnosis between UC and CD. There has been a growing interest in the use of cross-sectional imaging modalities such as magnetic resonance enterography (MRE), ultrasonography (US), and computed tomography (CT) as tools to supplement endoscopy in the diagnosis and monitoring of IBD [18]. These techniques are instrumental in detecting mural and extramural complications and assessing laminal inflammation in areas affected by CD in the small bowel that are beyond the reach of colonoscopy [19]. Due to their ability to diagnose CD with high accuracy, cross-sectional imaging modalities are used to make differential diagnoses in suspected cases of UC [20]. This aspect is critical because these diseases differ in their prognosis and required treatments.

Although imaging techniques offer highly accurate IBD diagnosis, they require experienced personnel, sophisticated instruments, and high costs, hampering their routine application. Laboratory testing's advantage lies in the fact that these tests can be standardized, rapid, and cost-effective, but they can also be applied to the already established patient sample libraries to process independent investigations. An increasing number of laboratory tests, combined with endoscopy or imaging, are used to monitor disease activity or diagnose suspected IBD cases. As good laboratory test results rely on the proper use of molecular biomarkers from the patients' tissue, blood (serum), or fecal samples.

2. Non-Invasive Molecular Biomarkers of IBD

Biomarkers play critical roles in the early detection and monitoring of disease progression and therapeutic responses. Disease activity can be monitored with laboratory tests that measure circulating biomarkers in the blood (serum or plasma), tissue, or feces. A biomarker is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention" [21]. Identifying a biomarker or several biomarkers of a given condition's pathologies might help to diagnose, prognose, and assess therapeutic responses. For a biomarker to be effective, it should possess several attributes, such as being non-invasive, inexpensive, convenient for sampling, reproducible, and disease-specific (i.e., accurate and precise). An ideal biomarker also needs to have a rapid test-to-result turnaround time, be standardizable to provide comparable test results across different assays, be widely available and stable for storage, have a wide dynamic range, use defined thresholds to determine the absence/presence or extent of inflammation, and be responsive to changes in the state of inflammation [22].

2.1. Serum Biomarkers

Several inflammatory serum biomarkers have become part of routine laboratory testing for the diagnosis of IBD. Although they are not specific to IBD, these serum biomarkers are commonly used for initial diagnosis due to their ease of use, low cost, and well-established protocols. The most common of these tests are those for C-reactive protein (CRP) and the erythrocyte sedimentation rate (ESR).

CRP is a pentameric protein that is produced in the liver by hepatocytes. It is found in serum at <1 mg/L under physiological conditions. Its concentration increases during an acute-phase response, as pro-inflammatory cytokines such as IL-6, tumor necrosis factor α (TNF-α), and IL-1β stimulate its production in the hepatocytes [23][24][25]. CRP has a relatively short half-life (about 19 h) [26], making it a better indicator of inflammation than most acute-phase proteins. Elevated CRP levels are observed in most active CD cases, whereas the CRP levels of UC patients show little-to-no increase in the case of active disease [25][27]. This may reflect the production of CRP by mesenteric adipocytes in patients with CD [28]. Although CRP is widely used as a biomarker for IBD, it lacks specificity; elevated CRP levels are also observed in autoimmune disorders, infections, and malignancies [23].

ESR is a measure of how quickly erythrocytes sediment through plasma in a column, with a higher rate taken as indicating more inflammation. ESR values are affected by physiological factors such as pregnancy, age, and

gender, as well as changes in hematocrit levels in patients with anemia and polycythemia [29]. Medications that cause changes in the size of erythrocytes can also affect ESR values [30]. Changes in ESR values are not specific to IBD, and can be due to any inflammatory stimulus. Unlike CRP, ESR values are altered in both UC and CD, and it cannot distinguish them. ESR values peak more slowly than CRP, and take longer to return to normal after the end of an inflammatory flare [26].

CRP and ESR have been studied long enough to become established in IBD diagnosis. While both tests lack the specificity and accuracy to be considered a gold-standard diagnosis, CRP has some advantages over ESR. For example, the CRP concentration changes faster than the ESR value upon a change in disease activity, CRP has a broader range of abnormal values than ESR, and (unlike ESR) CRP does not show age-related variation [31].

Leucine-rich alpha-2 glycoprotein (LRG) is a 50 kD protein that is secreted by hepatocytes, neutrophils, macrophages, and intestinal epithelial cells [32][33][34]. It has recently emerged as a novel serological biomarker for IBD and rheumatoid arthritis. Studies have found that levels of LRG are elevated in patients with active UC, and decrease with a decline in disease activity [35][36]. Notably, elevated levels of LRG correlate better than CRP with clinical and endoscopic scores in patients with active UC and CD [36][37][38]. LRG has been also found to predict mucosal healing in both UC and CD patients with normal CRP levels [39].

2.2. Serological Antibodies

Serological testing is a well-established diagnostic tool for a variety of immune diseases. Its use in IBD has been mainly focused on patients with a confirmed diagnosis; little work has been done on its potential as a primary diagnostic tool in patients with suspected IBD. Perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCAs) and anti-*Saccharomyces cerevisiae* antibodies (ASCAs) are the two primary antibodies currently examined in IBD studies. ANCAs are a group of antibodies produced against antigens in the cytoplasm of neutrophils. ASCAs are produced against mannan and other yeast cell wall components. Both have been reported to provide clinically useful positive or negative predictive values: p-ANCA+/ASCA− is reported in patients with UC, while p-ANCA−/ASCA+ is seen in patients with CD. Although each of these biomarker antibodies can be used to discriminate UC from CD, they both have low accuracy and sensitivity [40]. Positive results for either antibody are not unique to IBD, and may be related to several other GI and inflammatory conditions, such as celiac disease, Behcet's disease, cystic fibrosis, and rheumatoid arthritis [40][41].

2.3. Fecal Biomarkers

Fecal biomarkers are the proteins that are explicitly found in stool samples of patients with IBD. The fecal biomarkers for IBD reported to date are mainly fecal leukocyte proteins. These include calprotectin, calgranulin C, lactoferrin, and lipocalin-2. They have several advantages over blood biomarkers, including the ease of sample accessibility, high biomarker concentration due to the direct contact of the fecal sample with the site of inflammation, and higher specificity for IBD because they reflect GI inflammation (unlike serum biomarkers, which are increased by various types of inflammation) [42].

Calprotectin is the most widely used fecal biomarker for IBD. It is a calcium- and zinc-binding protein that is abundant in neutrophils, eosinophils, and macrophages. Changes in its concentration are observed in various secretory and excretory products in the body upon activation of granulocytes and mononuclear phagocytes [43]. Elevated fecal calprotectin levels are expected in patients with active IBD, due to the presence of a high number of neutrophils in the GI tract, which is characteristic of the disease [26]. Calprotectin is resistant to degradation, and is stable for 7 days in fecal samples stored at room temperature [44]. Changes in fecal calprotectin levels are not exclusive to IBD; alterations are also observed in various colon and intestine diseases [45].

Calgranulin C (S100A12) belongs to the S100 family of low-molecular-weight calcium-binding proteins, which activate the NF-κB pathway and increase cytokine release during pro-inflammatory processes [29]. The serum concentration of calgranulin C is high in IBD [46], but the fecal concentration is higher, making the fecal assay more sensitive to IBD. Elevated levels of calgranulin C have been reported in other inflammatory conditions, such as arthritis [47].

Lactoferrin is another biomarker whose levels are significantly elevated in active IBD. It is an iron-binding glycoprotein that is found specifically in neutrophils; in this respect, it contrasts with calprotectin, which is found in several types of cells. Lactoferrin has high specificity and sensitivity for diagnosing active IBD [48].

Lipocalin-2 (LCN-2), also known as neutrophil gelatinase-associated lipocalin (NGAL) or siderocalin (Scn), is a bacteriostatic protein stored in neutrophil granules [49][50]. LCN-2 is involved in innate immunity by secluding iron from pathogenic bacteria, limiting their invasion. It is a highly stable protein whose elevated expression by gut epithelial cells has been demonstrated in colonic biopsies from inflamed areas of patients with IBD. Serum LCN-2 has been proven to be an active biomarker in UC patients, and it is widely used as a fecal biomarker of acute inflammation in the animal model of UC, indicating that it can potentially be used as a fecal biomarker of human UC. Upregulation of LCN-2 is believed to be induced by IL-22 and IL-17A [51].

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