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EDITORIAL

New strategies for colorectal cancer screening

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Abstract

Colorectal cancer (CRC) is still one of the leading causes of cancer-related death in Western countries, despite major improvements in its treatment. The dramatically high social and economic impact of CRC on human health makes the identification of a reliable screening tool of paramount importance. Current screening methods, such as the fecal occult blood test and colonoscopy do not adequately meet the ideal requisites of a screening test because, even if they are effective, they are limited first by too low specificity and sensitivity, or second by high invasiveness, costs and risk. Nowadays extended efforts are made by researchers to look for more reliable and effective screening tests based on a systems biology approach, using biological samples easily available, such as urine, breath, serum and feces. The effectiveness and reliability of several new attempts to screen these patients by non-invasive analysis of their biological samples using genomic (genetic and epigenetic alteration), transcriptomic (miRNA), proteomic (cancer-related antigens, new antibodies against tumor-associated antigens, mutated proteins) and metabolomic (volatile organic metabolites) methods are discussed in this review. Among the most interesting new screening tools, fecal fluorescent long-DNA, fecal miRNA and metabolomic evaluation in breath and/or serum seem to be most promising.

Key words: Colorectal cancer; Screening; Fecal occult blood test; Systems biology; Metabolomics; Proteomic; microRNA; Volatile organic compounds

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TRADITIONAL APPROACH TO COLORECTAL CANCER SCREENING

Colorectal cancer (CRC) is the second most commonly diagnosed cancer and the second leading cause of cancer death in Europe, with an incidence of 43 600 new cases between 2007 and 2008[1]. The dramatically high social and economic impact of CRC on human health makes the identification of a reliable screening tool of paramount importance. CRC, as a cancer actually fulfills the World Health Organization conditions required for mass screening, since it is a very common disease, with major morbidity and mortality rates and is almost always preceded by a slow progressive premalignant lesion (the adenomatous polyp) which can readily be removed leading to true cancer prevention[2]. Screening strategies for CRC involve the separation of the population into two main categories: average risk and high risk populations. Each of these categories is targeted using a different screening program. In the first group, adults over 50 years without a personal or family history of CRC, polyps or inflammatory bowel diseases (IBD) are screened. The high risk population includes subjects with a family history of CRC, a personal history of CRC or polyps or are index cases affected by IBD. There is, however, a third category, more specifically characterized by an heredofamilial risk and represented by hereditary cancer syndromes such

as familial adenomatous polyposis and hereditary non-polyposis CRC[3,4]. Such cases should be screened directly with total colonoscopy (TC). The average risk population reflects the vast majority of the population and needs to be screened by less-invasive, low-cost techniques with acceptable patient compliance[5]. For that reason, in the last decade, there has been a great interest and research effort in developing the optimal CRC screening tool.

Clinically validated screening strategies currently available in practice include fecal occult blood testing (FOBT), TC, flexible sigmoidoscopy (FS) and radiographic imaging, such as double contrast barium enema and virtual TC. FOBT is the most commonly used method for CRC screening. In this respect, it is non-invasive, inexpensive and matches patient compliance better than other screening tools. In 2008, Hewitson *et al*[6] published a systematic review comparing the results of four randomized controlled trials, using FOBT as a screening tool, and in approximately 320 000 patients screened, there was an overall reduction of the relative risk of dying of CRC of 16%. Despite this, FOBT has demonstrated an unacceptably low specificity rate. To improve its reliability in this regard, fecal immunohistochemistry testing (FIT), which specifically detects non-degraded human globin using anti-human hemoglobin antibodies, has replaced the older guaiac-based FOBT (which identified the heme group by pseudoperoxidase). Despite this major improvement, the search for occult blood in the feces still has severe limitations as a screening tool, mainly because of its low specificity, hence leading to a high number of unnecessary colonoscopies[7,8]. FS has been proposed as a balance between the invasiveness of a given test (such as low invasive tests like FOBT and FIT), their accuracy and their potential complications (*e.g.*, TC), considering that about two-thirds of the screened CRCs detected are located in the rectum and sigmoid colon. It may be possible to increase the performance characteristics of FS by combining it with FOBT/FIT, however, the risk of leaving undetected CRC in other colonic sites is currently unacceptable[9,10].

TC still remains the gold standard for the diagnosis of both colorectal polyps and malignancies. The National Polyp Study demonstrated that the incidence of CRC was reduced from 76% to 90%[11] after polypectomy. Although very effective for diagnosis and treatment, TC has the limitations of low patient compliance, high cost, a high level of invasiveness and a moderate incidence of serious complications in specific subgroups (an incidence of 0.1%-0.3% of life-threatening complications including bleeding and perforation). TC colonography (or virtual TC) involves the use of helical TC to generate high-resolution 3D images of the abdomen and pelvis, replacing the older barium enema in providing full structural evaluation of the entire colon. A study conducted by Fenlon *et al*[12] in a high risk population, reported a sensitivity of 71% for TC colonography, although this was strongly influenced by polyp size where only 55% of polyps between 1 and 5 mm in maximal diameter were correctly identified. The sensitivity for virtual diagnosis was significantly higher when polyps ranged between 6 and 9 mm or were larger than 10 mm in size (82% and 91%, respectively; $P = 0.001$)[12]. This investigation, however, had the drawbacks of considerable exposure to ionizing radiation, discomfort of the bowel preparation and the necessity to complete the procedure by TC in cases of polyp or cancer detection, as well as being expensive (with inherent derivative costs) and currently not suitable for screening purposes.

From these considerations it is clear that current screening methods do not properly meet the ideal requisites of a screening test, so that extended effort has been dedicated by researchers at looking for more reliable and effective screening tests based on the systems biology approach using biological samples easily available such as urine, breath, serum and feces. Since the human genome was completely identified in 2003, the entire set of genes and proteins expressed have been extensively studied using genomic, transcriptomic or proteomic approaches.

GENOMIC APPROACH TO CRC SCREENING

Several authors have attempted to identify cancer-related mutated DNA/RNA, mutated proteins or normal proteins abnormally synthesized [*e.g.*, carcinoembryonic antigen (CEA), cytokeratins] in different biological samples as potential biomarkers for CRC. Colorectal carcinogenesis is characterized by genetic alteration (gene mutation or gene amplification) and epigenetic alteration (gene *hypermethylation* or *chromatin* modification), which both transform normal epithelial cells into cancer cells. CRC cells are continuously shed in the feces, due to a high proliferative rate, so that mutated DNA can be readily detected in the feces of these patients. This issue is complex, where mutation in the *APC*, *K-ras* and *p53* genes were initially investigated in stool samples of CRC patients, in accordance with the Volgenstein model of *CRC* genesis[13]. Other markers have also been studied by Imperiale *et al*[14] who conducted a large population-based study comparing the fecal DNA test with FOBT, using a DNA marker panel formed by 21 mutations and demonstrated a sensitivity of 52% for invasive cancers compared with 13% for FOBT in the same population. Fecal DNA testing has been commercially available in the United States since 2003, but so far has rarely been adopted for screening despite preliminary studies showing that the use of a large pool of genetic markers results in a sensitivity of 71%-91% and a specificity of more than 93%[15]. A recent interesting approach involves the use of fluorescent long DNA (FL-DNA) measurement, designed to identify cancer DNA fragments greater than 150-200 db pairs. Changes are noted since cancer cells do not undergo apoptosis, which in normal epithelial cells typically initiate DNA cleavage and degradation producing small measurable fragments. This FL-DNA technique has shown a performance sensitivity up to 80% in detecting CRC[16]. Such mutated DNA can also

be demonstrated in the urine of CRC patients. Human urine has been shown to contain two types of DNA: large type, greater than 1 kb, presumably derived from cells shed into the urine from the urinary tract and small type, between 150 bp and 250 bp, derived from the circulation, which can cross the renal barrier. Sample urine collection is non-invasive and isolation of DNA from urine is easier than from others specimens, due its low extraneous protein content. The comparison of mutated *K-ras* sequences, in particular the mutation in codon 12, between tumor, blood and urine from CRC patients and healthy controls showed an 83% correspondence of mutated DNA in urine and tumor tissue in the same patients[17]. Epigenetic changes which characterize CRC cells have only been studied in urine samples; most notably, the *hypermethylated vimentin* (*m-VIM*) gene. The detection of *m-VIM* in urine samples is significantly associated with CRC when compared with healthy controls[18].

TRANSCRIPTOMIC APPROACH TO CRC SCREENING

The most recent transcriptomic approach to identify potential biomarkers for CRC involves the study of microRNAs (miRNA), short non-coding 18-22 nucleotide RNA molecules involved in regulation of gene expression through post-transcriptional processing. Their expression is deregulated in cancer cells where altered miRNA expression leads to altered expression of their target gene including a range of potential oncogenes and oncosuppressors during carcinogenesis. Chen *et al*[19] showed that levels of miRNA in the serum are stable, reproducible and consistent in humans, concluding that they can be potential biomarkers for different diseases. Recent studies have indicated that circulating microRNAs incorporated into microvesicles and exosomes may be involved in genetic informational exchange between cells and may regulate extracellular matrix degradation, immunologic response and angiogenic factors which favor cancer cell growth and metastasis[20]. MiR-145, miR-143, miR-135a and b, miR-17-92, miR-21 have been most studied in CRC where Ng *et al*[21] were able to identify a significant increase of miR92 in the plasma of CRC patients compared with controls. Similar results have been reported by Huang *et al*[22] demonstrated a significant increase in miR29a and miR92a in patients with adenomas and CRC compared with controls, supporting the hypothesis that the miR17-92 cluster could have a role in cell proliferation, tumor angiogenesis and apoptotic suppression. Altered miRNA[23] expression has been examined in the stools of CRC patients and could represent an optimal screening tool for this cancer where colonic cancer cells exfoliate in greater quantity and their nucleic acid can be extracted and distinguished from those of bacteria. In this regard, Link *et al*[24] compared fecal specimens of patients with CRC, patients with adenomas and normal controls, showing a specific miRNA pattern in the three groups where miR21, miR106 were over expressed in CRC patients compared with controls, but where levels were higher in patients with adenomas and tended to decrease in cancer cases. Other researchers, however, were unable to confirm the higher expression of miR21, whilst the clusters miR17-92 and miR135 have been found to be significantly higher in the feces of CRC patients when compared with controls[25]. Another fecal mRNA frequently investigated as a potential CRC marker in stool is the prostaglandin-synthase 2, which showed a sensitivity between 50% and 90% and a specificity of 93% or higher in the diagnosis of CRC, although the reliability of this study was limited by the small number of CRC patients evaluated[26,27].

PROTEOMIC APPROACH TO CRC SCREENING

A further method for early detection and screening of CRC is to look at the modified "proteome" as a direct effect of mutated gene expression or as the occurrence of new antibodies against tumor-associated antigens (TAAs) identified in CRC. Hundt *et al*[28] have published a systematic review of 19 studies, in which 52 protein markers were analyzed, using common standard procedures such as enzyme-linked immunoassay, radioimmunoassay or more recent approaches like chromatographic and mass spectrometric assays based on surface-enhanced laser desorption/ionization time-of-flight (TOF) and matrix-assisted laser desorption/ionization TOF technologies. These compounds can be divided into antigens, antibodies, cytokines and other CRC-relevant proteins. CEA is the most investigated marker. High CEA levels are derived from embryonic tissues and CRC, but they also increase in other malignancies, including gastric and pancreatic cancer, as well as in IBD and in smokers. Its role for screening is limited because CEA evaluation has been shown to have a sensitivity of only 43%-69% in detecting early CRC, whilst its reliability increases in metastatic cancer where assessment lies outside the screening purpose. Carbohydrate antigens such as CA 19-9, CA195, CA 50 or CA 72-4 have been investigated in many studies, but with comparatively disappointing results. The best performance amongst these antigens is that of CA 19-9, with a sensitivity ranging between 18% and 65% and a specificity of over 90%. Other antigens considered for screening purposes include the sialylated Lewis antigen X, CO 29.11[29], urokinase-type plasminogen activator[30] and small intestinal mucin antigen[31], but none of these serological antigens have so far demonstrated an acceptable reliability in clinical testing. Recently Matsubara *et al*[32] studying the proteome of CRC patients compared with healthy controls, using label-free quantitative mass spectrometry and protein microarray, identified the adipophilin or adipose differentiation-related protein, a protein involved in the cancer pathway and normally expressed in cancer cells but not by the normal mucosa. This protein has been investigated as a potential plasma biomarker for early CRC stages, showing high receiver

operating characteristics[32].

Other studies have focused on the use of autoantibodies antibodies against TAAs as serological markers for cancer diagnosis, because they are absent in healthy subjects and other non-cancer conditions. Many autoantibodies against known or unknown TAAs, have been found in the sera of patients with a range of malignancies[33-35]. Various technologies such as serologic analysis of recombinant cDNA expression libraries, first described in 1995 by Sahin *et al*[36], and protein arrays or phage display techniques have been used in their measurement. The occurrence of several serum autoantibodies against TAAs, such as epithelial cell adhesion molecule or cytokeratin, p53, p62, CEA, HER-2/neu, Ras, topoisomerase Ⅱ-alpha, histone deacetylases 3 and 5, ubiquitin C-terminal hydrolase L3, tyrosinase, tropomyosin and cyclin B1 have all been evaluated in CRC patients[37], but were detected only in a limited proportion of patients (< 40%). Mutated or abnormal proteins have been detected also in the feces as potential biomarkers for screening, including tumor pyruvate kinase type M2, which has good sensitivity for CRC (85%), but not for adenomas (28%)[38], S100 calcium binding protein A12 and metallopeptidase inhibitor 1. The latter showed a sensitivity for cancer of around 85% and a specificity of 95%[39] compared with healthy controls.

METABOLOMIC APPROACH TO CRC SCREENING

More recently, the study of specific metabolomic biomarkers for cancers has developed as a new frontier in cancer screening. Metabolomics are the endpoint of the "omics" cascade and incorporate the comprehensive study of low-molecular-weight metabolites, using high-throughput technologies, such as gas chromatography-mass spectrometry, or other analytical platforms. Ikeda *et al*[40] investigated the differences in serum metabolite profiles of esophageal, gastric and CRC patients and healthy volunteers, using the metabolomic approach to determine specific metabolomic biomarker candidates. They showed a different distribution of L-alanine, glucuronic lactone and L-glutamine in CRC patients, with a sensitivity of 54.5%-81.8% and a specificity of 6.7%-91.6%[40]. Specific metabolomes can be identified in several types of biologic samples, including feces, urine, serum, sputum and breath. In this regard, breath analysis could be considered the favored option for medical diagnostic purposes mostly because of its non-invasive nature, its low cost and its ready patient compliance[41]. Volatile organic compounds (VOCs) in exhaled breath were first isolated by Pauling *et al*[42] in 1971, and alteration in VOC production in cancer patients has been postulated to relate to (per)oxygenation of cell membrane-based polyunsaturated fatty acids resulting from genetic and/or protein mutations within tumor cells and the increased relative prevalence of reactive oxygen species within cancer cells[43,44]. Urine and serum are ideal tools for metabolomic analyses. Some studies using high-throughput techniques and artificial neural network statistics have identified some volatile organic metabolites as potential biomarkers for CRC in urine[45], and very recently, a Japanese group has developed a CRC-prediction model based on serum metabolomic analysis and which demonstrated a high sensitivity (82.8%) as a novel potential screening test for CRC[46]. A similar metabolomic approach was carried out by our group[47], looking at the VOCs contained in breath. In this study, 15 of the 58 VOCs identified formed a specific pattern in CRC patients and, using a probabilistic neural network, the ability to identify CRC patients showed a sensitivity of 86%, a specificity of 83% and an accuracy of 85% (area under the receiver operating characteristics curve: 0.85) for the diagnosis of CRC.

In conclusion, despite their usefulness and effectiveness, traditional methods for CRC screening are still far from fulfilling the optimal requisites for a screening test. The FOBT/FIT both have too low a sensitivity or specificity whilst the high sensitivity of CT is counterbalanced by its invasiveness and high cost. TC colonography is still improving its technical performance but is expensive and, in cases of positivity, a traditional TC is still required to remove polyps or for biopsies. New hopes are rapidly growing in this field with the application of the systems biology approach using biological samples which are readily available. Among these, the search for fecal FL-DNA, fecal miRNA and metabolomic evaluation in the breath and/or serum seems to be the most promising.

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