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Clinical applications of DNA methylation biomarkers in colorectal cancer

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KEYWORDS: biomarkers ■ colorectal cancer ■ DNA methylation ■ epigenetics ■ epigenomics

Colorectal cancer (CRC) is a common disease that results in significant morbidity and mortality worldwide. CRC is the second leading cause of cancer mortality worldwide, and accounts for over 600,000 deaths annually [1]. Biomarkers are molecules or substances found in blood, other bodily fluids or tissues that reflect a particular biological or pathological state. Blood- or stool-based biomarkers have the potential to revolutionize CRC diagnostics by providing noninvasive, patient-friendly and cost-effective early detection of CRC. Cancer biomarkers may have broader applications, including disease risk analysis in otherwise healthy people, molecular classification, therapy response prediction, disease prognostics and recurrence monitoring in patients who have been treated for cancer. In recent years, abnormal patterns of DNA methylation have emerged as candidate cancer biomarkers [2]. Here, we will summarize recent advances in the use of DNA methylation biomarkers in CRC clinical applications.

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Germline mutations in hereditary CRC syndromes have proven valuable in identifying individuals at high risk of developing CRC, enabling personalized surveillance and early intervention. These classic high-risk alleles are mostly caused by loss-of-function coding-region mutations. By contrast, germline polymorphisms in regulatory regions of the *MLH1* gene have been found to result in a higher likelihood of epigenetic silencing from the affected *MLH1* allele, thereby increasing the risk of developing

mismatch-repair-deficient CRC [3,4]. In rare cases, the effect of the polymorphism is strong enough to cause constitutional silencing of the *MLH1* allele [3], which would be detectable in a blood-based *MLH1* DNA methylation assay. Another example of a systemic epigenetic alteration is loss of imprinting of the *IGF2* gene, which results in the activation of the normally silent allele of the *IGF2* gene, an epigenetic phenomenon associated with various types of cancer, including CRC. Systemic loss of imprinting can be determined in peripheral blood and may be associated with an increased risk of developing CRC [5].

Epigenetic biomarkers have also been explored as cost-effective and noninvasive alternatives to colonoscopy for the early detection of CRC. An ideal early detection DNA methylation biomarker would be present in the targeted malignancy at high frequency and detectable in bodily fluids or secretions despite the presence of background DNA from normal tissues. The most recent DNA methylation biomarker studies show promising results that have the potential to impact the way CRC patients will be treated in the future. The field of DNA methylation biomarker research is evolving rapidly and organized efforts are being made to structure biomarker research and development [6]. In the past, however, many studies have been performed without employing clear rules of evidence, using small sample sizes and relying on candidate gene approaches and non-quantitative detection methods. It has long been known that aberrant patterns of DNA methylation from CRC cells can be detected in tumor-derived cell-free DNA (cfDNA) found in plasma, serum or feces of cancer patients [7]. It is thought that cfDNA in cancer patients is a result of direct shedding from the primary

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tumor through apoptosis, necrosis or secretion, or potentially originates from free circulating tumor cells or metastatic cell deposits [7]. Studies have reported the existence of specific hypermethylated genes in blood and stool of patients with CRC and their associations with clinical disease and prognosis [8–10]. It has not yet been determined whether the use of a blood-based test or a stool-based test for early CRC detection is more efficient and more accepted by the public. DNA methylation biomarkers for early detection of CRC have been studied in both blood and feces. A blood-based test has the advantage that it can be used for primary detection, as well as postoperative follow-up for local and distant recurrences. In addition, the processing of a relatively small amount of blood may be preferred in laboratories above handling bulky feces. However, stool-based biomarkers may have some advantages over blood-based tests. Because colorectal adenomas and early CRCs are situated in the inner lining of the colorectum and are in direct contact with feces, tumor secretions and shed cells mix directly with colorectal content. Although it is difficult to estimate whether the half-life of tumor DNA is longer or shorter in feces than in blood, it seems conceivable that the concentration of tumor-derived DNA is higher in the intestine than in the bloodstream. Indeed, reported sensitivities for blood-based CRC DNA methylation biomarkers range up to 90% with a specificity of 88% [11]. In stool, sensitivities of 95% with specificities of 94% have been published [12]. A recent study by Ahlquist *et al.* reported a DNA stool test that detects methylated *BMP3*, *NDRG4*, vimentin and *TFPI2*, mutant *KRAS*, the *ACTB* gene and the quantity of hemoglobin [13]. This assay detected adenomas larger than 2 cm with a sensitivity of 82% and CRC with a sensitivity of 91% at a specificity of 93%.

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Little is known about the exact mechanism of tumor cfDNA derivation and spread and its relationship to biomarker detection. Factors that might influence diagnostic performance of a CRC DNA methylation biomarker include tumor, host and test characteristics. It is likely that tumor biology affects detectability. For instance, CpG island methylator phenotype (CIMP) tumors harbor high frequencies of

DNA methylation and could therefore be more amenable to detection using DNA methylation biomarkers compared with tumors with lower frequencies of aberrant DNA methylation (as is the case for rectal tumors). Furthermore, the total amount of DNA shed by the tumor is likely to influence test performance. In general, biomarker studies show that stage IV CRC tumors, which usually have a higher total tumor burden, are detected more frequently than stage I CRC tumors or adenomas. Although it is known that cfDNA concentrations can vary significantly between individuals, the exact reason why is not clear. Besides individual variances in tumor biology, cfDNA spread and clearance by the liver and kidneys, as well as the wide-ranging diversity of the way samples are treated in different laboratories, may contribute to this variation. With the very low concentrations of tumor-derived DNA in the bloodstream, the volume of blood used in the analysis will affect assay sensitivity. Indeed, most DNA methylation-based detection assays use volumes in the ml range, rather than the μ l range [2]. For example, the recently commercialized DNA methylation marker *SEPT9* is assayed in 4 ml of plasma and yields a sensitivity of 90% for the detection of CRC at a specificity of 88% [11,14]. Methylated *SFRP2*, on the other hand, was reported to detect 67% of CRCs at a specificity of 94% using only 200 μ l of serum [15]. In a recently published study we demonstrated that methylated *THBD* detects 74% of stage I/II CRCs at a specificity of 80% using 1 ml of serum [16]. It is difficult to compare these markers without considering the large difference in volume. It is possible that *SFRP2* or *THBD* would outperform *SEPT9* at equal volumes. Future studies will have to point out if increasing test volume also increases sensitivity without loss of specificity.

Molecular classification of CRC is possible using various molecular traits identified in CRC tumor samples. Some commonly used classifications are microsatellite instability (MSI), mutations of the *TP53*, *KRAS* and *BRAF* genes. Although chromosomal instability status also reflects a molecular characteristic and is associated with worse prognosis in CRC, it is usually not used as a molecular classifier owing to its heterogeneity and poorly defined characterization. CIMP is seen in a subset of CRC tumors with unusually frequent DNA hypermethylation of a defined set of loci, and was first described in colon cancers more than a decade ago [17]. Although the exact

molecular mechanisms and causes of CIMP are unknown, this phenomenon in CRC is associated with a specific phenotype (older age, female sex, family history of CRC, proximal location in the colon, mucinous cell differentiation, specific precursor lesions and smoking) and is tightly associated with sporadic MSI, *BRAF* and *KRAS* mutations and *MLH1* promoter methylation [18,19]. CIMP is a CRC classifier, but also has prognostic meaning, and has been studied as a predictive marker in CRC. A prognostic biomarker gives information on tumor biology; a predictive marker tells us how a specific tumor responds to drug treatment. Although studies have suggested that MSI as well as CIMP have a disadvantageous effect on adjuvant fluorouracil (5-FU)-based treatment in CRC patients (predictive), in general MSI⁺CIMP⁺ status is associated with better clinical outcome (prognostic) [20]. It is difficult to study the predictive value of MSI or CIMP on 5-FU drug response due to the high degree of overlap between CIMP and MSI and the fact that 5-FU is the most widely used chemotherapy, administered to almost all eligible CRC patients.[21,22]. Recently, Ebert *et al.* published a specific association of a particular promoter methylation status with therapy response. Hypermethylation of *TFAP2E* in primary as well as metastatic CRCs was shown to be associated with decreased expression of this gene

and nonresponsiveness to 5-FU. The fact that hypermethylation of *TFAP2E* was observed in 51% of the patients in this cohort indicates the possible impact this knowledge could have on future CRC treatment. In addition, if this gene was not methylated, it resulted in a six-times higher response rate than the total CRC population [23].

The clinical application of DNA methylation biomarkers in CRC is rapidly developing into a competitive scientific field and has been noticed by the commercial industry as a marketable product. We expect DNA methylation biomarkers to take a definitive place in CRC diagnostics in the near future.

Financial & competing interests disclosure

PW Laird is inventor of the MethyLight technology, which has been licensed exclusively to Epigenomics AG, for use by Epigenomics in colorectal cancer DNA methylation biomarker assays, including SEPT9. PW Laird receives royalties in this context, but he is neither a shareholder nor consultant for Epigenomics AG, and receives no other financial support from Epigenomics AG. Epigenomics AG did not contribute to this manuscript in any way. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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