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Abstract

Aberrant promoter methylation, an 'epigenetic' form of genomic instability that leads to transcriptional silencing of tumor suppressor genes, is increasingly being recognized as a crucial component in the evolution of human cancers. With our limited knowledge of the molecular basis and timing of the initiation of altered methylation events in the stepwise progression of cancers, the biggest challenge we currently face is to identify novel biomarkers and technologies for the timely screening of patients carrying such alterations. One such strategy would be to develop tests for the detection of fecal DNA methylation patterns that will improve the sensitivity of noninvasive screening tests for colorectal neoplasia, and moreover, will decrease both mortality and the incremental costs of treating colorectal cancers.

KEYWORDS: fecal DNA, colorectal cancer, methylation, epigenetics

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Review

Detection of Fecal DNA Methylation for Colorectal Neoplasia: Does It Lead to an Optimal Screening Test?

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Aberrant promoter methylation, an 'epigenetic' form of genomic instability that leads to transcriptional silencing of tumor suppressor genes, is increasingly being recognized as a crucial component in the evolution of human cancers. With our limited knowledge of the molecular basis and timing of the initiation of altered methylation events in the stepwise progression of cancers, the biggest challenge we currently face is to identify novel biomarkers and technologies for the timely screening of patients carrying such alterations. One such strategy would be to develop tests for the detection of fecal DNA methylation patterns that will improve the sensitivity of noninvasive screening tests for colorectal neoplasia, and moreover, will decrease both mortality and the incremental costs of treating colorectal cancers.

Key words: fecal DNA, colorectal cancer, methylation, epigenetics

A promising noninvasive screening tool for various human malignancies including colorectal neoplasia is to assay for molecular biomarkers that represent a 'specific' or 'spectrum' of genetic and/or epigenetic alterations. In this context, fecal DNA testing based on genetic alterations has been an area of active investigation since 1992, and has gradually evolved into the development of multi-target DNA assays [1, 2]. Despite the enthusiasm in this approach for investigating genetic mutational signatures, up till now there have only been 2 large-scale studies that have reported data on fecal-based testing [3]. The successful extrapolation of such a strategy

is extremely complex and difficult to achieve due to the challenges it poses to fecal DNA testing based upon the detection of epigenetic alterations, such as DNA methylation [4, 5]. Nonetheless, the interest in this field is immense, and the past several years have seen an explosion in investigations dealing with the identification of epigenetic markers in cancer [6–8]. To reap the full potential of this methodology, investigators have based their assays on the unique differences in methylation patterns between the 'cancerous'- and 'normal'-appearing tissues, differences that form the basis for the development of noninvasive biomarkers to detect the presence of tumors in blood, sputum, urine, and stool samples [5, 9]. To test the validity and usefulness of fecal-based epigenetic marker screening, human colorectal cancer has provided a good model for investigating whether DNA methylation can be adopted as an optimal diag-

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nostic screening test, since several other noninvasive screening tools including fecal occult blood testing (FOBT) and fecal DNA testing based on genetic alterations are currently the subjects of active research.

However, in contrast to the relatively well accepted mutation-based fecal DNA testing [1-3], methylation-based testing has been initiated more recently, and is beginning to identify adequate specific markers that are representative of epigenetic 'signature' alterations. All of these investigations will be useful, as in the long run, the challenges to discovering new specific markers and technologies will not only improve noninvasive screening tests, but will also help reduce both overall patient mortality and the incremental costs of treating colorectal neoplasia.

Fecal Occult Blood Testing (FOBT) and Colonoscopies

Human colorectal cancer has emerged as an experimental model for the introduction of innovative and effective cancer screening and early detection tools in large populations because advanced malignant tumors in the colon are accompanied by a significantly high rate of mortality. The problem is further compounded when weighed in economic terms, as the longer persistent treatment that is necessary when there is a failure to diagnose these lesions early leads to substantive incremental treatment costs. FOBT can reduce colorectal cancer mortality by 15%-33% through the early detection and removal of adenomatous polyps and cancers by colonoscopies or surgical operations [10-15]. Even though FOBT has a specificity of 95%, the usefulness of FOBT is somewhat limited due to its lack of sensitivity, which only ranges somewhere between 15-30% [16], especially for advanced adenomas [17, 18]. Similarly, even though colonoscopy has a sensitivity of over 90%, and ~99% specificity for the detection of adenocarcinoma and advanced adenomas, this procedure suffers from the practical limitation that it requires bowel preparation and sedation, which causes patient discomfort and a small risk of serious medical complications [16, 19-23]. An optimal screening test is defined as one that is easy to conduct, noninvasive, less expensive, and acceptable to the larger popula-

tion [24]. Thus, the challenge is to tailor a non-invasive screening strategy that could be employed for the detection of colorectal neoplasia in its early stages, thus eliminating the need for other expensive and invasive surgical procedures.

Fecal DNA Testing Based on Genetic Alterations

Since it was concluded that no single 'specific' gene mutation can be identified and universally attributed to all colorectal cancers, it became necessary to use larger panels of markers targeted for certain mutations to identify a specific human malignancy [2, 3, 25]. One example of such multi-target DNA testing is the strategy that can detect up to 21 specific mutations in the *adenomatosis polyposis coli* (*APC*), *p53*, *K-ras* gene, together with a microsatellite instability marker (*BAT-26*), and long-fragment DNA. Most reports using this assay have been based on an approach developed either by EXACT Sciences (Maynard, MA, USA) or using the commercially available PreGen-Plus (Lab Corp, Burlington, NC, USA) test. However, these assays require a large volume of fecal sample from which purified DNA is prepared using oligonucleotide-based hybrid capture [3]. An initial small number of pilot studies reported that this approach had a sensitivity that varied from 62-91% for cancer detection, and 27-82% for adenomatous polyps, with a high degree of specificity for subjects without colorectal neoplasia ranging from 93-98% [2, 25-27]. Similarly, a subsequent larger multi-center study (evaluating 4,404 subjects) using this panel reported a sensitivity of 52% for invasive cancers and 18% for advanced neoplasms, with a specificity of 94% for subjects with negative findings on colonoscopy (Table 1) [3].

Fecal DNA Testing Based on Epi-genetic Alterations

More recently, there has been growing interest in extending this methodology to another possible DNA-based target, *i.e.*, the detection of aberrant DNA hypermethylation of CpG islands in the genes [4, 5, 28]. Epigenetic alterations are defined as heritable signatures of information other than nucleotide sequences. In mammals, almost all 5-methylcytosine

(m5C) sequences occur in the 5'-CpG-3' dinucleotide sequences that appear at a relatively low frequency in the genome [29]. Nevertheless, several clusters of CpG sites are present in the genome, and these clusters of CpG sites are defined as CpG islands [30]. CpG islands are typically unmethylated under normal conditions, except for the imprinted regions of one parent allele and inactive X chromosome [31–33]. In cancerous cells, CpG islands within gene promoters are recognized as crucial components of cancer initiation and progression because tumor-suppressor genes, such as *CDKN2A* and *BRCA1*, are inactivated by hypermethylation in their promoter CpG islands [34]. Methylation analysis of a number of gene promoters in DNA from stool samples has been less comprehensively investigated, but has been suggested to be a sensitive diagnostic tool for colorectal tumors [35–40]. Table 2 shows summarized data from all the reports to date on fecal DNA testing targeted for epigenetic alterations. The pilot studies reporting fecal DNA testing on epi-genetic alterations had a sensitivity varying from 42% to 77% for cancer, and 31% to 55% for adenomas, and an overall specificity ranging from 63% to 100% for subjects without colorectal neoplasia. Compared with multi-target DNA testing, fecal DNA methylation testing has traditionally exhibited slightly lower sensitivity and specificity. However, the majority of these pilot studies were limited to analysis of a single epigenetic biomarker analogous to fecal DNA testing of a specific genetic alteration. Such an approach is inherently flawed, because unlike genetic alterations, which can be more precise and may involve a specific

gene/loci, the biological significance of methylation changes is probably greater when they are evaluated using the multiple biomarkers that are required to enhance the overall sensitivity and specificity of these assays. This use of multiple biomarkers is especially important because several genes are frequently methylated in both colorectal cancers and premalignant adenomas relative to the lower frequency of methylation that can be detected in their counterpart normal-appearing mucosa [41–43]. This might be one of the reasons for the observed decreased specificity of fecal DNA methylation testing in comparison to conventional tests that involve the evaluation of a specific 'genetic' alteration. Despite the fact that normal mucosa displays some degree of methylation, there is still a significant opportunity to design an assay that can distinguish patients with neoplasia from those without neoplasia using a selected panel of genes that have a consider-

Table 1 Colorectal Neoplasia Detection by Multi-target DNA Testing PreGen-Plus in Stool

Reference	Sensitivity, % (n)		Specificity*, % (n)
	Cancer	Adenoma	
Ahluquist <i>et al.</i> [2]	91 (20/22)	82 (9/11)	93 (26/28)
Tagore <i>et al.</i> [25]	63 (33/52)	57 (16/28)	98.2 (111/113)
Brand <i>et al.</i> [26]	69 (11/16)	–	–
Calistri <i>et al.</i> [27]	62 (33/53)	–	97 (37/38)
Imperiale <i>et al.</i> [3]	52 (16/31)	18.2 (76/418)	94.4 (1344/1423)

*Specificity for subjects without colorectal neoplasia.

Table 2 Colorectal Neoplasia Detection by Fecal DNA Testing based on Epigenetic alterations in Stool

Reference	Biomarker	Sensitivity, % (n)		Specificity*, % (n)	Method
		Cancer	Adenoma		
Chen <i>et al.</i> [40]	<i>Vimentin</i>	46 (43/94)	–	90 (178/198)	MSP
Muller <i>et al.</i> [35]	<i>SFRP2</i>	77 (10/13)	–	77 (10/13)	Methylight
Lenhard <i>et al.</i> [39]	<i>HIC1</i>	42 (11/26)	31 (4/13)	100 (32/32)	MSP
Petco <i>et al.</i> [38]	<i>CDKN2A & MGMT</i>	–	55 (16/29)	63 (12/19)	MSP
	<i>CDKN2A</i>	–	31 (9/29)	84 (16/19)	
	<i>MGMT</i>	–	48 (14/29)	72 (13/18)	
	<i>hMLH1</i>	–	0 (0/29)	90 (17/19)	

*Specificity for subjects without colorectal neoplasia.

able difference in the degree and frequency of methylation.

Seeking Tumor-specific 'Hot Spots (/markers/ biomarkers)' of Aberrant Methylation

Aberrant DNA methylation is often mistakenly considered to be uniformly spread around the core region of the promoter with a CpG island. In reality, however, the methylation pattern is not homogeneous among various CpG sites within a CpG island. For instance, the *hMLH1* gene, which is one of the major DNA mismatch repair genes, has a large CpG island within its promoter that clearly divides it into at least 2 discrete regions of methylation (Fig. 1). Deng *et al.* examined the methylation status of 3 regions (A, B and C) in the *hMLH1* promoter, and compared the methylation status to the gene expression in 24 cell lines [44]. They concluded that only the C region was associated with the loss of gene expression, an observation that was later confirmed by a large cohort of clinical colorectal cancer cases [45, 46]. In particular, the methylation in regions A and B occurs in normal mucosa, but the methylation in region C occurs only in tumor tissues and is

considered to be "tumor-specific" [46, 47]. This concept added a new dimension to the interpretation of promoter hypermethylation data, suggesting that the methylation of a 'tumor-specific' region rather than the mere 'presence' of methylation is a key determinant of the biological meaning of these findings. This finding arises another question: Even if the tumor-specific sites are selected as biomarkers for screening fecal DNA testing, are these real tumor-specific markers?

Belshaw *et al.* reported that, using a nested methylation-specific PCR (MSP) assay (Fig. 1), virtually all DNA specimens, irrespective of whether these were extracted from tumors, normal mucosa, or stool, displayed frequent hypermethylation in *ESR1*, *MGMT*, *HPP1*, *CDKN2A*, *APC* and *hMLH1* gene markers [36]. However, this confusing scientific scenario was rescued when a less sensitive semi-quantitative assay, a modified combined bisulfite restriction analysis (COBRA), was employed, and all of these DNA samples were re-analyzed. It was interesting that using such a methodology, significant differences in degrees of methylation in *ESR1* and *MGMT* were detected between stool specimens and their counterpart normal mucosa specimens. These

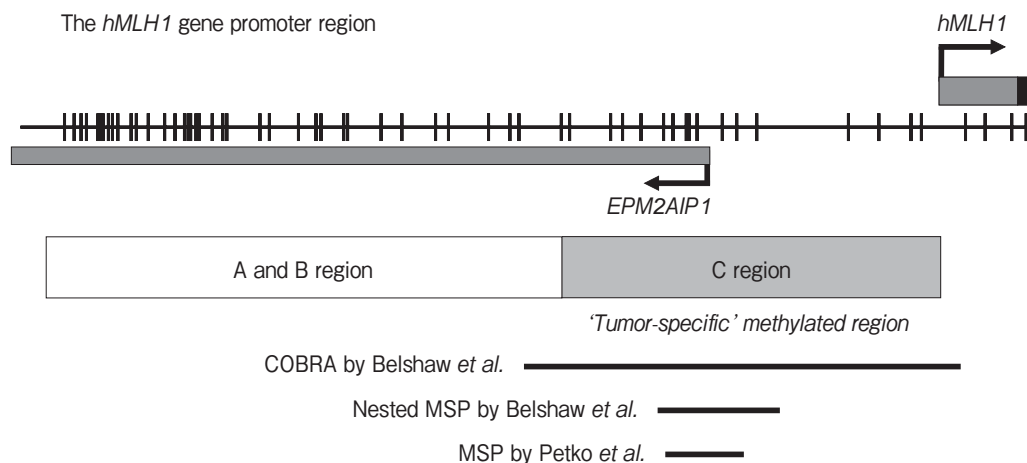


Fig. 1 Regions within the *hMLH1* promoter.

In the *hMLH1* promoter region, the *EPM2AIP1* gene is also located opposite of the *hMLH1* gene. Arrows indicate the transcriptional start site of both genes. The non-coding region and coding region in exon1 are indicated by gray and black squares, respectively. The CpG site is shown by a vertical line. Regions A, B and C are defined by Deng *et al.* The regions A and B are methylated in normal colorectal mucosa, and the methylation of this region is not affected by the expression of the gene. Region C is considered to include the 'cancer-specific' methylation sites. The locations of MSP, nested MSP, and the COBRA assay used by fecal DNA testing are also shown in this Figure.

differences were previously not evident when using the traditional highly-sensitive MSP assay. In this study, the region of the *hMLH1* promoter analyzed was also located within the C region (Fig. 1), which is a “tumor -specific” region. All materials, including stool specimens from patients with and without neoplasia, displayed hypermethylation of *hMLH1* when analyzed by nested-MSP but did not show hypermethylation when analyzed by the modified COBRA. On the other hand, Petko *et al.* [38] reported that, by using a traditional MSP, the specificity of *hMLH1* assay was 90% (Table 2), and the analyzed region of

hMLH1 promoter was similar to that of Belshaw *et al.* (Fig. 1). The only conclusion that can be drawn regarding the observed differences in the methylation status of the *hMLH1* promoter C region between these pilot studies is that the differences relate to the differences in the sensitivity of the assays.

Both the MSP and COBRA methods use bisulfite-modified genomic DNA as a PCR amplification template (Fig. 2) [48, 49]. Bisulfite modification converts cytosine to uracil at an efficiency $\geq 99\%$; however, the rate of conversion of methylated cytosine to uracil is very slow [50]. Thus, bisulfite-modified

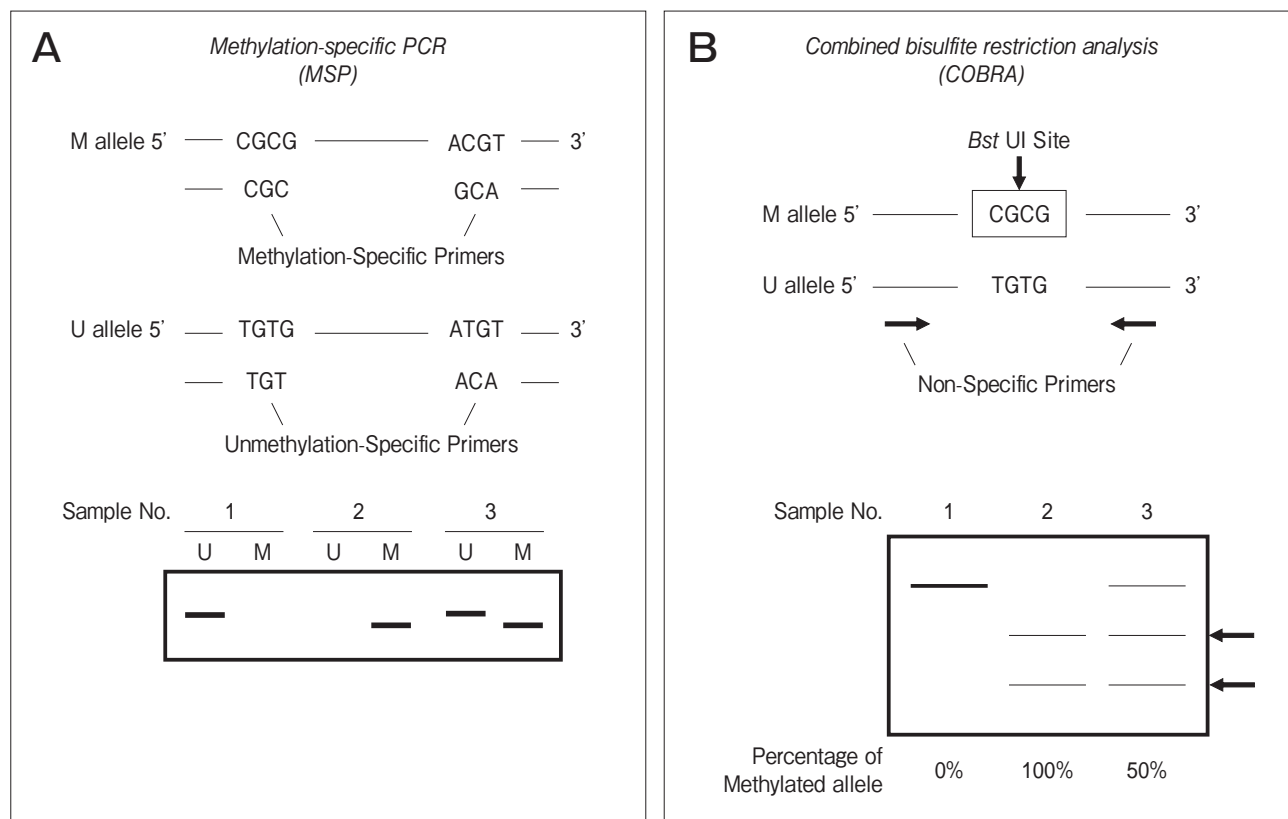


Fig. 2 Schema of MSP and COBRA.

A: Methylation-specific PCR (MSP). Genomic DNA was chemically modified by sodium bisulfite. This modification generates a methylation-dependent sequence difference at CpG sites by converting unmethylated cytosine residues to uracil when the CpG is not methylated, while methylated cytosine residues are retained as cytosine when the CpG is methylated. Methylation-specific primers hybridize to the methylation-specific sequence. On the other hand, unmethylation-specific primers hybridize to the unmethylation-specific sequence. Thus one could detect methylation by the existence of PCR product.

B: Combined bisulfite restriction analysis (COBRA). The PCR primers for COBRA are designed to hybridize to the sequence with both methylated and unmethylated cytosines. By use of a restriction enzyme that includes the CG sequence within its recognition sequence, one could anticipate the level of methylated allele in a quantitative manner. Arrows indicate methylated alleles cut by *Bst*UI (recognition sequence; CGCG).

DNA displays unique differences between unmethylated cytosine and methylated cytosines, which are subsequently used for the detection of methylated versus unmethylated alleles. The MSP assay can detect as little as 0.1% methylation, while COBRA, being less sensitive, can detect as few as 0.5% methylated alleles. The nested MSP is an even more highly sensitive assay, and can detect a single methylated allele present in 50,000 unmethylated alleles (0.002%) [51]. The pilot studies suggest that 0.002–0.1% of methylated DNA encoding the C region of *hMLH1* promoter can be detected from the DNA extracted from exfoliated epithelial cells present in the total fecal mass.

In this context, another interesting finding was reported in the study by Chen *et al.* [40], who reported a dense CpG region located upstream of the first two-thirds of the first exon of vimentin gene, which is normally unmethylated, but becomes methylated in colorectal cancers. Interestingly, the vimentin gene is typically unmethylated and not expressed in normal colorectal epithelial crypt cells. This would mean that the expression of this gene is not under epigenetic control. In addition, in order to identify the cancer-specific 'hot spots' of DNA methylation sites within the vimentin gene promoter, several MSP primer sets were analyzed. As anticipated, only 2 of these several sets of MSP primers were available for further screening.

At present, only a small number of studies have been done on fecal DNA testing for investigating epigenetic alterations, but still these initial pilot studies provide some clues and insight into the future design of newer technologies that will come into existence based upon the knowledge gained from these experiments. At least we have reached a certain degree of consensus on some of the previously less-understood paradigms; first, it has been clear that cancer-specific methylation sites definitely exist within gene promoters and could be used to distinguish patients with colorectal neoplasia from subjects without neoplasia, even in a non-invasive test such as methylation detection in stool samples; second, the methylation status of CpG sites that do not influence the associated gene expression can become the basis for developing new biomarkers for screening. In reality, as many as half of such genes have CpG islands within their coding regions or introns [30], a fact

that distinguishes them from conventional tumor suppressor genes, which primarily have CpG sequences in the promoter regions only.

Conclusion

Thanks to the completion of the human genome project, we can utilize information about the specific arrangements of the conventional 4 nucleotide bases, adenine, cytosine, guanine and thymine. However, additional information that affects phenotypes is stored in the distribution of a modified base, 5-methylcytosine. As of now, our understanding of 5-methylcytosine is still in its infancy. It is hoped that in the next few decades, with the completion of the "epigenome" project, we will acquire a better understanding of the distribution of 5-methylcytosine in various cell types, including normal and neoplastic cells, that will help improve future strategies for the early detection of cancers. It goes without saying that the completion of such a project would provide seminal insights into the complexities of the methylation machinery that is believed to be an underlying 'epigenetic signature' harbored in a large majority of human cancers.

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