Promising DNA methylation markers for specific and sensitive detection of colon cancer in blood

INTRODUCTION:

Colorectal cancer (CRC) is the fourth leading cause of cancer-related death in the Western world. In the U.S. and EU there are >400,000 new cases and >180,000 deaths attributable to CRC each year. Less than 40% of CRCs are diagnosed at a localized stage when CRC is the most treatable. Early stage CRC often has no symptoms, highlighting the need for screening age-appropriate, asymptomatic individuals. Colonoscopy is the gold standard screening method but this procedure is invasive, costly and not readily accessible or acceptable to a large percentage of age-eligible individuals. Fecal occult blood testing (FOBT) is non-invasive and inexpensive but suffers from lack of sensitivity and specificity. As data demonstrating the clinical and cost-effectiveness of CRC screening has emerged, the impetus for improving non-invasive testing options has intensified. A blood-based screening test would overcome many of the practical disadvantages of current options and, when coupled with good sensitivity and specificity, would become an excellent means for identifying individuals for colonoscopy. OncoMethylome Sciences is developing a sensitive and non-invasive, plasma-based test for colorectal cancer screening, especially in the non-symptomatic at-risk population, such as people above the age of 50.

MATERIALS AND METHODS:

Marker identification: Using epigenetic re-expression profiles of colon cancer cell lines, candidate genes were identified and the most promising markers were tested on tissue using the Base5 methylation profiling platform (1). Promoter sequences were linked with gene expression to identify epigenetically silenced genes. An established pharmacologic unmasking strategy (5-aza-2'-deoxycytidine [DAC] and trichostatin A [TSA]) for re-expression analysis of epigenetically targeted genes was combined with proprietary advanced bioinformatics tools to identify genes prone to promoter methylation.

Marker selection in colon tissue: Marker candidates identified by re-expression were screened using 37 real-time Methylation-Specific PCR (real-time MSP) assays. These assays were used to assess the methylation status of 29 genes in 293 formalin-fixed paraffin-embedded (FFPE) tissue samples collected from various clinics. Samples included 99 colon adenocarcinomas of various stages, 16 adenomas, 63 samples from patients with cancer other than CRC, 39 samples from patients with no evidence of cancer and 76 distant resection ends (histopathologically normal) from CRC patients. These samples were divided into training and independent test sets, and used to select the gene methylation markers best able to discriminate between cancerous and non-cancerous samples.

Plasma sample collection and preparation: A standardized screening trial (The Netherlands) was initiated in 2006. In this trial, asymptomatic subjects aged 50 or above are screened with colonoscopy, FOBT and real-time MSP using DNA from stool and blood. The trial is expected to enroll 1600 individuals within 3 years. In addition, prospectively collected plasma samples from multiple centers (Germany and The Netherlands) were used. In these trials, symptomatic patients, attending a Gastroenterology clinic and ultimately diagnosed with CRC, provided a plasma sample for use

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in real-time MSP. From the ongoing trials, 317 plasma samples were available for the present study. Four main categories of plasma samples were used: 86 samples with no suspicious findings, 39 adenomas, 50 samples from patients with cancers other than CRC, and 120 samples from patients covering all stages of CRC. The case report forms of the remaining 22 individuals were not yet available for analysis. Blood was collected using EDTA Vacutainer™ tubes.

DNA preparation and processing: DNA was isolated from tissue and plasma, using standard DNA isolation methods and equipment, and bisulphite modified using a commercially available kit. Analyte quantitations were done using real-time MSP for each colon gene assay.

RESULTS:

Assay validity rate in tissue and plasma:
293 FFPE and 317 plasma samples were processed using real-time MSP (Table 1). The real-time MSP assays produced valid results in 98% of the FFPE samples and in 100% of the plasma samples.

Sample sets	Sample types	Sample numbers	Valid tests [%]
Tissue training set	CRC	65	65/65 [100]
	Controls	76	74/76 [97]
	Total	141	139/141 [99]
Tissue test set	CRC	34	34/34 [100]
	Controls	39	39/39 [100]
	Other Cancers	63	59/63 [94]
	Adenomas	16	16/16 [100]
	Total	152	148/152 [97]
Tissue sets combined	CRC	99	99/99 [100]
	Controls	115	113/115 [98]
	Other Cancers	63	59/63 [94]
	Adenomas	16	16/16 [100]
	Total	293	287/293 [98]
Plasma training set (1)	CRC	42	42/42 [100]
	Controls	34	34/34 [100]
	Other cancers	25	25/25 [100]
	Total	101	101/101 [100]
Plasma training set (2)	CRC	78	78/78 [100]
	Adenoma	39	49/49 [100]
	Controls	52	64/64 [100]
	Other cancers	25	25/25 [100]
	Unknown	22	22/22 [100]
	Total	216	216/216 [100]
Plasma sets combined	CRC	120	120/120 [100]
	Adenoma	39	49/49 [100]
	Controls	86	98/98 [100]
	Other cancers	50	50/50 [100]
	Unknown	22	22/22 [100]
	Total	317	317/317 [100]

Table 1: Summary of samples tested by real-time MSP and validity rate

Marker selection in colon tissue: Based on re-expression, 224 different gene assays representing 145 genes were tested on the Base5 methylation profiling platform (data not shown, see reference 1 for details). The 37 most differentially methylated gene sequences assessing 29 genes were validated on retrospectively collected tumors from 65 colorectal cancer patients (all stages) and 74 distant resection ends (histopathologically normal) using real-time MSP. Several markers reliably differentiated CRC from normals in those tissue samples. The results were confirmed on an independent test set containing 59 samples from patients with cancer other than CRC, 39 non-cancerous controls, 34 colon adenocarcinomas and 16 adenomas. Several combinations of the tested markers reliably detected CRC with high specificity and sensitivity (data not shown here, see reference 2 for additional details).

Marker testing in plasma: The best performing markers in tissue were assessed on 101 plasma samples from multiple centers (plasma training set 1). These plasma samples included 34 samples with no suspicious findings, 25 samples from patients with cancers other than colon cancer and 42 samples from patients covering all stages of CRC, with 50% representing early stage disease (stages I and II). Several of the tested markers detected CRC in those plasma samples (data not shown). The best performing genes were selected to confirm the results with an independent sample set prospectively collected from multiple centers (plasma training set 2). Reducing the number of gene assays applied to plasma training set 2 resulted in fewer assays per sample and a greater aliquot of plasma DNA was added per PCR reaction. The 216 plasma samples of training set 2 included 52 samples with no suspicious findings, 39 adenomas, 25 samples from patients with cancers other than colon can-

cer and 78 samples from patients covering all stages of CRC, with 42% representing early stage disease (stages 0, I and II). The case report forms of the remaining 22 individuals were not yet available for analysis. The performance of the best marker combination to reliable detect CRC using 1-4 mL of plasma is shown in Table 2. As can be seen in Table 3, sensitivity with the same panel of genes was improved by using a minimum starting plasma volume of 2.0 mL.

Sample groups (plasma training set 2)	Plasma gene panel: OSMR, GATA5, NDRG4 and ADAM23			
	Optimized for sensitivity		Optimized for specificity	
	Sensitivity % (# detected / # total) [95% CI]	Specificity % (# detected / # total) [95% CI]	Sensitivity % (# detected / # total) [95% CI]	Specificity % (# detected / # total) [95% CI]
Early stages CRC: 0, I, and II	70% (23/33) [54-86]		58% (19/33) [41-75]	
All stages CRC	73% (57/78) [63-83]		64% (50/78) [53-75]	
Adenomas	10% (4/39)		5% (2/39)	
Controls		92% (6/77) [86-98]		99% (1/77) [96-100]

Table 2: Performance characteristics of the plasma gene panel using real-time MSP and plasma volumes of 1-4 mL

Sample groups (plasma training set 2)	Plasma gene panel: OSMR, GATA5, NDRG4 and ADAM23			
	Optimized for sensitivity		Optimized for specificity	
	Sensitivity % (# detected / # total) [95% CI]	Specificity % (# detected / # total) [95% CI]	Sensitivity % (# detected / # total) [95% CI]	Specificity % (# detected / # total) [95% CI]
Early stages CRC: 0, I, and II	70% (23/33) [54-86]		58% (19/33) [41-75]	
All stages CRC	74% (54/73) [64-84]		67% (49/73) [56-76]	
Adenomas	10% (4/39)		5% (2/39)	
Controls		92% (6/77) [86-98]		99% (1/77) [96-100]

Table 3: Performance characteristics of the plasma gene panel using real-time MSP and plasma volumes of 2-4 mL

CONCLUSIONS:

- Initial work confirmed that a plasma-based assay employing the 4 methylation markers NDRG4, GATA5, OSMR and ADAM23 correctly identified patients with CRC.
- ALL CANCER STAGES: Performance characteristics of the 4 gene panel demonstrated 74% sensitivity / 92% specificity (optimized for sensitivity) and 67% sensitivity / 99% specificity (optimized for specificity).
- EARLY CANCER STAGES (0, I and II): Performance characteristics of the 4 gene panel demonstrated 70% sensitivity / 92% specificity (optimized for sensitivity) and 58% sensitivity / 99% specificity (optimized for specificity).
- A standardized, prospective protocol continues to collect plasma samples for further validation of this approach and refinement of the optimal methylation panel.

REFERENCES:

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2) Louwagie, J. et al, AB-29-AACRMD (2007): Feasibility of a DNA methylation assay for noninvasive CRC screening

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