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DNA Methylation Biomarkers for Blood-Based Colorectal Cancer Screening

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BACKGROUND: Sensitive, specific blood-based tests are difficult to develop unless steps are taken to maximize performance characteristics at every stage of marker discovery and development. We describe a sieving strategy for identifying high-performing marker assays that detect colorectal cancer (CRC)-specific methylated DNA in plasma.

METHODS: We first used restriction enzyme–based discovery methods to identify marker candidates with obviously different methylation patterns in CRC tissue and nonpathologic tissue. We then used a selection process incorporating microarrays and/or real-time PCR analysis of tissue samples to further test marker candidates for maximum methylation in CRC tissue and minimum amplification in tissues from both healthy individuals and patients with other diseases. Real-time assays of 3 selected markers were validated with plasma samples from 133 CRC patients and 179 healthy control individuals in the same age range.

RESULTS: Restriction enzyme–based testing identified 56 candidate markers. This group was reduced to 6 with microarray and real-time PCR testing. Three markers, *TMEFF2*, *NGFR*, and *SEPT9*, were tested with plasma samples. *TMEFF2* methylation was detected in 65% [95% confidence interval, 56%–73%] of plasma samples from CRC patients and not detected in 69% (62%–76%) of the controls. The corresponding results for *NGFR* were 51% (42%–60%) and 84% (77%–89%); for *SEPT9*, the values were 69% (60%–77%) and 86% (80%–91%).

CONCLUSIONS: The stringent criteria applied at all steps of the selection and validation process enabled successful identification and ranking of blood-based marker candidates.

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Of the new molecular approaches, the assessment of epigenetic events is one of the most promising means of identifying marker candidates for the early detection of cancer. The regulation of gene expression by aberrant methylation has been well characterized in tumor biology in general *(1, 2)* and has been extensively described for colorectal cancer (CRC)[6] in particular *(3–5)*. The development of blood-based cancer-detection tests should improve patient compliance and thereby increase the detection of disease at earlier stages. Increased concentrations of circulating methylated DNA have been reported in the blood of cancer patients *(6, 7)*; however, a routine process for validating epigenetic biomarkers has not been implemented. We previously proposed a systematic marker-identification process that identifies aberrantly methylated genes in cancer tissue and validates the results in plasma samples from affected individuals *(8)*. We have used CRC as a model for implementing this process and describe criteria that are necessary to ensure optimal biomarker performance. In particular, sensitivity and specificity must be optimized through the identification of markers that show the highest differences in methylation between the cancer and the background. This goal can be achieved by comparing the degree of DNA methylation in the target cancer with that in a healthy tissue sample from the same organ, healthy blood samples, and organs from which cells could be present in the bloodstream as a result of disease conditions associated with the tested population. Also extremely important is to develop high-performing assays capable of detecting extremely low amounts of methylated DNA in a very high background of unmethylated or partially methylated DNA. In this study, we describe the selection and validation of potential blood-based epigenetic biomarkers for early detection of CRC or for other clinical

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[6] Nonstandard abbreviations: CRC, colorectal cancer; PBL, peripheral blood lymphocytes; HM, heavy methyl; qPCR, quantitative real-time PCR; LOD, limit of detection; AUC, area under the ROC curve; bisDNA, bisulfite-modified DNA; PMR, percent-methylation rate.

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applications and report the performance of 3 of these markers in the analysis of plasma samples.

Materials and Methods

HUMAN SAMPLES

Written informed consent was obtained from all study participants, and the process adhered to local ethics guidelines. CRC tissue was obtained from surgical resections, fresh-frozen, and stored at −80 °C. Samples of healthy tissue were obtained at least 6 cm from a tumor and stored as described above. Plasma samples were obtained from healthy individuals and CRC patients a minimum of 1 week after colonoscopy. The clinical diagnosis of CRC was confirmed by histologic analysis, and colonoscopy examinations confirmed that healthy individuals had no colon-related disease (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol?/issue?).

BIOMARKER DISCOVERY

We used methylation-sensitive restriction enzyme–based discovery methods [methylation-specific arbitrarily primed PCR and methylated CpG island amplification (MCA) *(9, 10)*] to identify sequences that were differentially methylated in pathology-verified tumors by comparing the results with those obtained for healthy tissues and peripheral blood lymphocytes (PBLs) from people in the same age group. We extracted DNA from tissue samples with Genomic-tip 500/G columns (Qiagen) and used the DNA Isolation Kit I with a MagNA Pure LC System (Roche Applied Science) to extract DNA from PBLs *(11)*.

METHYLATION MICROARRAYS

We used microarrays as previously described to study diseased and healthy colon-derived tissues, other cancer tissues, and samples of healthy tissue from the same organs *(12)*. Probes were designed for the region identified in the discovery process (typically a fragment of 250–500 bp), and up to 1000 bp of flanking sequence was included if it was within a CpG island. DNA was extracted from tissues as described above. We designed 2 arrays, each with approximately 250 probes covering ≥54 different amplicons. The first array was interrogated with amplicons from 358 tissues (89 CRC samples, 55 colorectal polyps, 31 samples of inflammatory bowel disease, 116 extracolonic cancers, 29 healthy colon samples, 14 nonpathologic PBL samples, and 24 other noncolonic, nonpathologic samples). The second array was tested with 429 tissue samples (18 CRC samples, 311 extracolonic cancers, 34 nonpathologic PBL samples, and 66 other noncolonic, nonpathologic samples).

DEVELOPMENT OF QUANTITATIVE REAL-TIME PCR ASSAYS

MethyLight assays *(13)* were designed for regions identified in the discovery process and confirmed by microarray analysis. Assays for marker candidates identified from the literature were designed for the promoters or first exons of genes and were within CpG islands. Heavy methyl (HM) quantitative real-time PCR (qPCR) assays were designed as previously described *(14)*. We used CpGenome Universal Methylated DNA (MP Biomedicals) at concentrations between 31.6 μg/L and 31.6 mg/L for calibration curves. We evaluated the 90% limit of detection (LOD) for each assay with a dilution series of CpGenome Universal Methylated DNA in a background of 50 ng human genomic DNA (Roche Applied Science). The 90% LOD was defined as the lowest concentration of spiked methylated DNA in a background of 50 ng human genomic DNA for which the measurement values had an area under the ROC curve (AUC) of ≥0.9 compared with measurements without spiked methylated DNA.

qPCR ANALYSIS OF TISSUES

We obtained 114 CRC tissue samples, 51 samples of nonpathologic colon tissues that had been fresh-frozen at surgery, and 51 PBL samples from healthy individuals in the same age range as the patients. Surgical samples were verified for tumor content. We extracted DNA as in the biomarker-discovery process and treated the DNA with bisulfite according to a previously described protocol *(15)*. We quantified bisulfite-modified DNA (bisDNA) with a methylation-nonspecific assay for an intragenic region of the *GSTP1*[7] gene (glutathione S-transferase pi) and adjusted the DNA concentration to 10 ng/PCR reac-

[7] Human genes: *TMEFF2*, transmembrane protein with EGF-like and two follistatin-like domains 2; *NGFR*, nerve growth factor receptor (TNFR superfamily, member 16); *SEPT9*, septin 9; *GSTP1*, glutathione S-transferase pi; *VTN*, vitronectin; *GATA3*, GATA binding protein 3; *GDNF*, glial cell derived neurotrophic factor; *OPCML*, opioid binding protein/cell adhesion molecule-like; *PENK*, proenkephalin; *TFAP2A*, transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha); *APP*, amyloid beta (A4) precursor protein (peptidase nexin-II, Alzheimer disease); *CACNA1G*, calcium channel, voltage-dependent, T type, alpha 1G subunit; *HOXA1*, homeobox A1; *NEUROG1*, neurogenin 1; *APBA2*, amyloid beta (A4) precursor protein-binding, family A, member 2; *TRRAP*, transformation/transcription domain–associated protein; *BCOR*, BCL6 co-repressor; *CAV1*, caveolin 1, caveolae protein, 22kDa; *CD44*, CD44 molecule (Indian blood group); *CDH13*, cadherin 13, H-cadherin (heart); *VCAN* (formerly *CSPG2*), versican; *FCGR2A*, Fc fragment of IgG, low affinity IIa, receptor (CD32); *GSK3B*, glycogen synthase kinase 3 beta; *PCDH17*, protocadherin 17; *TAF11*, TAF11 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 28kDa; *EYA4*, eyes absent homolog 4 (Drosophila); *TUSC3*, tumor suppressor candidate 3; *ZDHHC22*, zinc finger, DHHC-type containing 22; *FOXL2*, forkhead box L2; *ALX4*, aristaless-like homeobox 4; *SIX6*, SIX homeobox 6; *BCL6*, B-cell CLL/lymphoma 6 (zinc finger protein 51); *DNAJC5*, DnaJ (Hsp40) homolog, subfamily C, member 5; *DUX2*, double homeobox, 2; *ESTG020896* (no HUGO ID available); *MSH6*, mutS homolog 6 (E. coli); *ESTG2308609* (no HUGO ID available); *DLX5*, distal-less homeobox 5; *NPBWR1* (formerly *GPR7*), neuropeptides B/W receptor 1; *APBA2*, amyloid beta (A4) precursor protein-binding, family A, member 2 (X11-like); *SLC32A1*, solute carrier family 32 (GABA vesicular transporter), member 1; *SLITRK1*, SLIT and NTRK-like family, member 1; *KCTD12*, potassium channel tetramerisation domain containing 12; *ONECUT2*, one cut homeobox 2; *RNF4*, ring finger protein 4; *SMAD7*, SMAD family member 7.

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tion for subsequent testing. Calibration curves were conducted for each assay as described above with CpGenome Universal Methylated DNA concentrations between 400 μg/L and 5 mg/L. We tested 10 ng DNA per reaction in duplicate for each assay on the LightCycler 2.0 (Roche Diagnostics) or the ABI Prism 7900 instrument (Applied Biosystems).

COLLECTION OF PLASMA SAMPLES

We chose a sample size of 150 for plasma samples from CRC patients to achieve confidence limits of ±8% around an assumed sensitivity of 50%. The targeted sample size for plasma samples from healthy individuals was 200 for confidence limits of ±5% around an assumed specificity of 90%. We obtained blood samples via phlebotomy into EDTA-containing Vacutainer tubes (BD Medical Systems). We separated the plasma by centrifugation (1500*g* for 10 min) within 4 h of blood collection and then carefully removed the buffy coat. The plasma was placed in a 15-mL tube and centrifuged at 1500*g* for 10 min. If we obtained more than 1 tube per patient, we pooled the plasma before storage at −80 °C.

METHYLATION ANALYSIS OF PLASMA DNA

Patient samples were randomized and blinded as to the clinical diagnosis. We prepared DNA by a 3-step process. First, we extracted free-floating DNA with the MagNa Pure LC Total Nucleic Acid Isolation Kit–Large Volume (Roche Applied Science) and the MagNA Pure System. We distributed 6 mL over 6 MagNA Pure wells and concentrated the eluates with Microcon filters (Millipore). We quantified the amount of genomic DNA recovered after extraction by means of a qPCR assay with primers that contained no cytosine residues (cytosine-free fragment, CFF1). Second, we treated the DNA with bisulfite and measured the DNA concentration with the bisDNA assay. Third, we measured the DNA concentrations (in milligrams per liter) of methylated *TMEFF2* (transmembrane protein with EGF-like and two follistatin-like domains 2), *NGFR* [nerve growth factor receptor (TNFR superfamily, member 16)], and *SEPT9* (septin 9) DNA for each sample by means of a single qPCR reaction for each HM assay and a calibration curve, as described above. All assays were run on the LightCycler 2.0 instrument. See Table 2 in the online Data Supplement for details regarding the assay components and running conditions for HM marker and control assays.

STATISTICAL ANALYSIS

We analyzed microarray and qPCR tissue data by using $\log_{10}$-transformed percent-methylation rates (PMRs) *(12)*. PCR analyses of plasma samples were based on $\log_{10}$-transformed DNA concentrations (as measured by the respective methylation-marker, bisDNA, or CFF1 assay) normalized to the plasma volume. Plasma samples with no measurable bisDNA were eliminated from analysis. We estimated DNA amounts for all qPCR assays from calibration curves by linear regression on the crossing points by the second-derivative maximum method *(16)*. Plasma DNA estimates from methylation-specific assays were scaled by the maximum fluorescence of the respective amplification curve relative to the maximum fluorescence of the calibrators. For tissue samples, analyses were based on the means of replicate marker measurements.

For univariate analyses, we estimated AUCs by means of the trapezoidal rule. The Wilcoxon–Mann–Whitney test was used for quantitative comparisons of 2 samples, the McNemar test was used to compare the accuracies of qualitative markers, and the Kruskal–Wallis test was used to compare results for disease stages. Logistic regression and the Wald test were used for multivariate panel analysis *(17)*. The predictive performance of each multivariate model is given as an AUC value corrected for overfitting bias and was calculated from the bootstrap-corrected concordance index (C index) *(18)*. All *P* values are 2-sided. Confidence intervals for proportions of amplified samples were set at 95% and based on binomial distributions.

Results

INITIAL SELECTION OF DIFFERENTIALLY METHYLATED MARKER CANDIDATES

We assessed >600 marker candidates identified in genome-wide discovery experiments with a previously described scoring system that accounts for the number of independent identifications of that marker during discovery, the location of the given marker within a CpG island or the promoter region of a gene, identification in multiple experiments, and other criteria *(12)*. Only markers with a score of at least 2 were advanced for further analysis.

Although sequences identified by methylation-specific arbitrarily primed PCR and methylated CpG island amplification indicated differentially methylated marker candidates, the statistical significance of differences between sample types cannot be evaluated with these methods. In addition, it is not technically possible to compare more than a few tissue types simultaneously. Therefore, we used DNA-methylation arrays and/or qPCR to quantify the extent of marker candidate methylation in different tissue types. Because of time and resource limitations, a subset of the candidates identified in the genome-wide discovery process (76 total) and 26 candidates identified from the literature were further validated in 2 separate methylation-microarray studies; 25 marker candidates are

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Table 1. Step-wise validation results for a selected subset of 25 marker candidates derived from the discovery process and from the literature.[a]

	Methylation array			Real-time PCR				
Gene symbol (HUGO)	AUC, CRC vs all controls	AUC, CRC vs colon controls	PBL: median PMR, %	PBL: median PMR, %	Healthy colon: median PMR (90% range), %	CRC: median PMR, (90% range), %	AUC	Exclusion criteria
BCL6	0.73	0.78	**25**					Failed chip-selection criteria
DNAJC5	0.67	0.73	**23**					
DUX2	0.75	0.70	**17**					
ESTG020896[b]	**0.64**	**0.63**	9					
MSH6[c]	**0.58**	**0.64**	0					
ESTG2308609[b]	**0.62**	**0.56**	**22**					
DLX5	0.84	0.83	0	**PBL+**				Failed PBL filter
NPBWR1 (*GPR7*)	0.73	0.82	0	**PBL+**				
APBA2[c]	NT[d]	NT	NT	**PBL+**				
SLC32A1	0.85	0.89	0	**1.0**				
SLITRK1	0.86	0.85	1	**0.3**				
KCTD12	0.67	0.77	0	0.0	0 (0–0)	**0 (0–28)**	**0.53**	Insufficient CRC methylation
ONECUT2	0.72	0.77	2	0.0	1 (0–1)	**0 (0–2)**	**0.24**	
RNF4	0.79	0.84	0	0.0	0 (0–0)	**0 (0–0)**	**0.52**	
SMAD7	0.68	0.71	0	0.0	0 (0–0)	**0 (0–1)**	**0.59**	
TUSC3[c]	0.82	0.74	1	0.2	3 (1–10)	11 (1–32)	0.71	Failed LOD test
TMEFF2	0.90	0.94	0	0.1	4 (1–9)	30 (1–81)	0.81	
ZDHHC22	0.88	0.87	9	0.0	0 (0–1)	3 (0–27)	0.78	HM assay failed
VTN	0.93	NT	1	0.0	2 (0–4)	20 (0–77)	0.81	Plasma positive
FOXL2	0.67	NT	0	0.0	NT	7 (0–38)	NT	Candidates for plasma testing
NGFR	0.83	0.87	0	0.0	0 (0–2)	3 (0–60)	0.69	
ALX4	0.65	0.83	0	0.1	1 (0–2)	11 (0–58)	0.74	
SIX6	0.77	0.85	0	0.0	0 (0–0)	16 (0–46)	0.83	
EYA4	0.66	0.81	4	0.0	2 (0–4)	26 (0–76)	0.88	
SEPT9	0.84	NT	0	0.0	2 (1–7)	26 (0–89)	0.87	

[a] A list with 31 additional candidates can be found in Table 3 in the online Data Supplement. Data for some markers have previously been reported [Model et al. (*12*)]. Results that led to the exclusion of a candidate gene are printed in boldface.
[b] No HUGO gene name or symbol available.
[c] Marker candidates derived from the literature.
[d] NT, not tested.

shown in Table 1 to illustrate the selection process for blood-based screening markers. A list with 31 additional candidates can be found in Table 3 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue2.

SELECTION CRITERIA FOR METHYLATION-ARRAY CANDIDATES

The DNA methylation–array data allowed us to rank marker candidates by their ability to differentiate neoplastic and control samples and by their methylation rate in PBLs. We used 3 criteria to select the best markers among the 45 array candidates for further investigation. First, discrimination between CRC and healthy colon tissue had to be significant with an AUC ≥0.70. Second, the differentiation of CRC and all other tissue samples had to be significant with an AUC ≥0.65. Third, PBL samples were required to have an absolute methylation rate of ≤10%.

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With these criteria, we increased the probability that our marker candidates obtained their hypermethylation signature during tumorigenesis, that the signature was reasonably specific for CRC, and that candidates would not be methylated in the target analyte.

Twenty-seven of the 45 marker candidates passed the testing with these initial selection criteria for informative DNA methylation–based biomarkers suitable for testing in the screening application (22 markers from the genome-wide discovery process and 5 from the literature). The *TMEFF2* marker displayed the best performance in the array study with regard to all of the selection criteria. The *SEPT9* and *VTN* (vitronectin) markers achieved scores comparable with *TMEFF2* (Table 1).

SELECTION OF MARKER CANDIDATES WITH SENSITIVE qPCR ASSAYS

Analyses of DNA-methylation microarrays allow the rapid quantification of methylation and ranking of marker candidates, but the analytical sensitivity of microarrays is reduced compared with PCR analysis. Moreover, arrays do not always generate candidates that show the consistent comethylation over several CpG positions that is required for qPCR assays. Therefore, we next developed qPCR assays (MethyLight or HM) *(13, 14)* for the 27 marker candidates selected from the array studies and assessed methylation with an independent sample set. We added 11 markers [*GATA3*, GATA binding protein 3; *GDNF*, glial cell derived neurotrophic factor; *OPCML*, opioid binding protein/cell adhesion molecule-like; *PENK*, proenkephalin; *TFAP2A*, transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha); *APP*, amyloid beta (A4) precursor protein (peptidase nexin-II, Alzheimer disease); *CACNA1G*, calcium channel, voltage-dependent, T type, alpha 1G subunit; *HOXA1*, homeobox A1; *NEUROG1*, neurogenin 1; *APBA2*, amyloid beta (A4) precursor protein-binding, family A, member 2; and *TRRAP*, transformation/transcription domain–associated protein on the basis of a study that indicated increased methylated DNA in CRC tissue *(19)*.

The qPCR assays possessed the following technical characteristics and fulfilled the requisite analytical-performance criteria. First, the maximum length of the amplicon was ≤150 bp. Second, the assay consistently detected 50 pg of completely methylated DNA. Third, the assay showed no detectable amplification of 50 ng of unmethylated DNA. Fourth, 50 pg of completely methylated DNA was consistently detected in a background of 50 ng of unmethylated DNA. Assay development was attempted for all 38 marker candidates. Nine candidates failed the initial criteria, such as assay length or unsuitable CpG site location for primer and probe placement [*BCOR*, BCL6 co-repressor; *CAV1*, caveolin 1, caveolae protein, 22kDa; *CD44*, CD44 molecule (Indian blood group); *CDH13*, cadherin 13, H-cadherin (heart); *VCAN* (formerly *CSPG2*), versican; *FCGR2A*, Fc fragment of IgG, low affinity IIa, receptor (CD32); *GSK3B*, glycogen synthase kinase 3 beta; *PCDH17*, protocadherin 17; and *TAF11*, TAF11 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 28kDa]. Of the remaining 29 assays, 22 passed analytical testing and were tested in studies of tissue samples.

For advancement of a marker candidate to plasma testing, we required the assay to fulfill the following

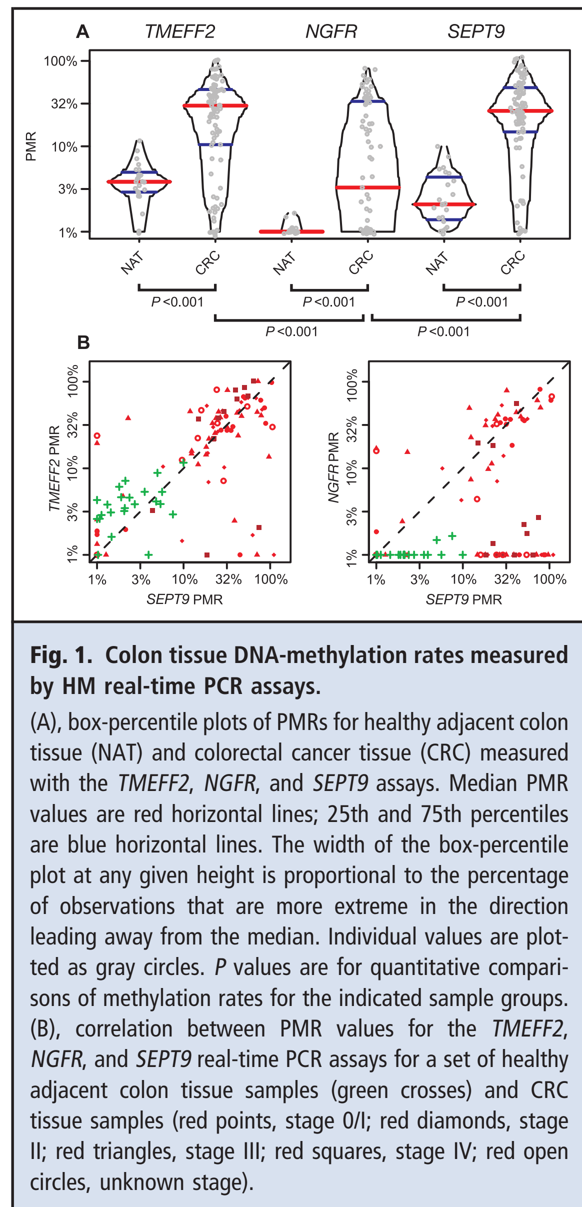

Fig. 1. Colon tissue DNA-methylation rates measured by HM real-time PCR assays.

(A), box-percentile plots of PMRs for healthy adjacent colon tissue (NAT) and colorectal cancer tissue (CRC) measured with the *TMEFF2*, *NGFR*, and *SEPT9* assays. Median PMR values are red horizontal lines; 25th and 75th percentiles are blue horizontal lines. The width of the box-percentile plot at any given height is proportional to the percentage of observations that are more extreme in the direction leading away from the median. Individual values are plotted as gray circles. *P* values are for quantitative comparisons of methylation rates for the indicated sample groups. (B), correlation between PMR values for the *TMEFF2*, *NGFR*, and *SEPT9* real-time PCR assays for a set of healthy adjacent colon tissue samples (green crosses) and CRC tissue samples (red points, stage 0/I; red diamonds, stage II; red triangles, stage III; red squares, stage IV; red open circles, unknown stage).

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Table 2. Marker panels.

Panel	Marker	Odds ratio[a] (95% CI[b])	*P*	Bias-corrected AUC
Tissue				
TMEFF2 + *NGFR*	*TMEFF2*	2.3 (1.5–3.6)	0.05	0.81
	NGFR	2.0 (1.4–3.0)	0.07	
SEPT9 + *NGFR*	*SEPT9*	4.6 (2.9–7.1)	0.0005	0.89
	NGFR	2.4 (1.6–3.7)	0.03	
SEPT9 + *TMEFF2*	*SEPT9*	3.8 (2.5–5.7)	0.001	0.87
	TMEFF2	2.5 (1.6–4.0)	0.05	
SEPT9 + *NGFR* + *TMEFF2*	*SEPT9*	4.3 (2.7–7.0)	0.003	0.89
	NGFR	2.4 (1.5–3.7)	0.06	
	TMEFF2	1.2 (0.7–2.2)	0.7	
Plasma				
TMEFF2 + *NGFR*	*TMEFF2*	1.4 (1.2–1.6)	0.004	0.74
	NGFR	1.7 (1.5–2.0)	0.0003	
SEPT9 + *NGFR*	*SEPT9*	2.6 (2.3–3.1)	<0.0001	0.81
	NGFR	1.4 (1.2–1.6)	0.04	
SEPT9 + *TMEFF2*	*SEPT9*	2.8 (2.4–3.2)	<0.0001	0.81
	TMEFF2	1.3 (1.1–1.4)	0.05	
SEPT9 + *NGFR* + *TMEFF2* + bisDNA	*SEPT9*	2.6 (2.2–3.1)	<0.0001	0.79
	NGFR	1.2 (1.0–1.5)	0.3	
	TMEFF2	1.2 (1.0–1.4)	0.4	
	bisDNA	1.0 (0.8–1.3)	0.9	

[a] Tissue odds are per 10-fold change in PMR; plasma odds are per 10-fold change in DNA concentration.
[b] CI, confidence interval.

biological criteria. First, differentiation of CRC tissue from healthy colon tissue had to be significant with an AUC ≥0.70. Second, PBLs were required to have an absolute methylation rate of ≤0.2%. The requirement for distinguishing CRC tissue from healthy colon tissue is identical to the selection criterion for first microarray and confirms that the markers were successfully transferred to the qPCR platform. The low-methylation requirement for PBLs was made more stringent by approximately 2 orders of magnitude because of the high analytical sensitivity of the qPCR assays. To facilitate faster screening of candidate markers, we applied a simplified qualitative version of the PBL criterion to some assays by requiring no amplification in 5 replicate measurements of 50 ng of PBL DNA.

We tested marker candidate assays in 2 tissue studies. The first study consisted of 28 CRC samples, 46 samples of healthy colon, and 25 PBL samples. The second study consisted of 86 CRC samples, 23 healthy colon samples, and 26 PBL samples. Assays for markers *TMEFF2* and *NGFR* were run in both studies. The results of the first study have previously been reported and are summarized in Table 1 with the results of the second study *(12)*. Methylation-rate distributions for selected candidate markers are shown in Fig. 1A.

On the basis of the results for the tissue samples, 10 of the 22 qPCR marker assay candidates fulfilled the sensitivity and specificity criteria and were advanced to plasma testing. Hypothesizing that a higher methylation rate in cancer tissue implies a higher concentration of methylated target molecules in the bloodstream, we further ranked the candidate assays by median methylation rate in tumor tissue. *TMEFF2*, *SEPT9*, *EYA4* [eyes absent homolog 4 (Drosophila)], and *VTN* were clearly superior with ≥20% methylation in >50% of the CRC samples.

We carried out multivariate logistic regression analysis for all possible 2- and 3-marker combinations to try to improve our ability to differentiate control and CRC samples. As we previously have observed *(12)*, markers were hypermethylated in a common subset of CRC samples (88% of CRC samples with a PMR of >10% for any marker) and showed a typical CpG island methylator phenotype *(20)*. *TMEFF2* and *SEPT9* showed very

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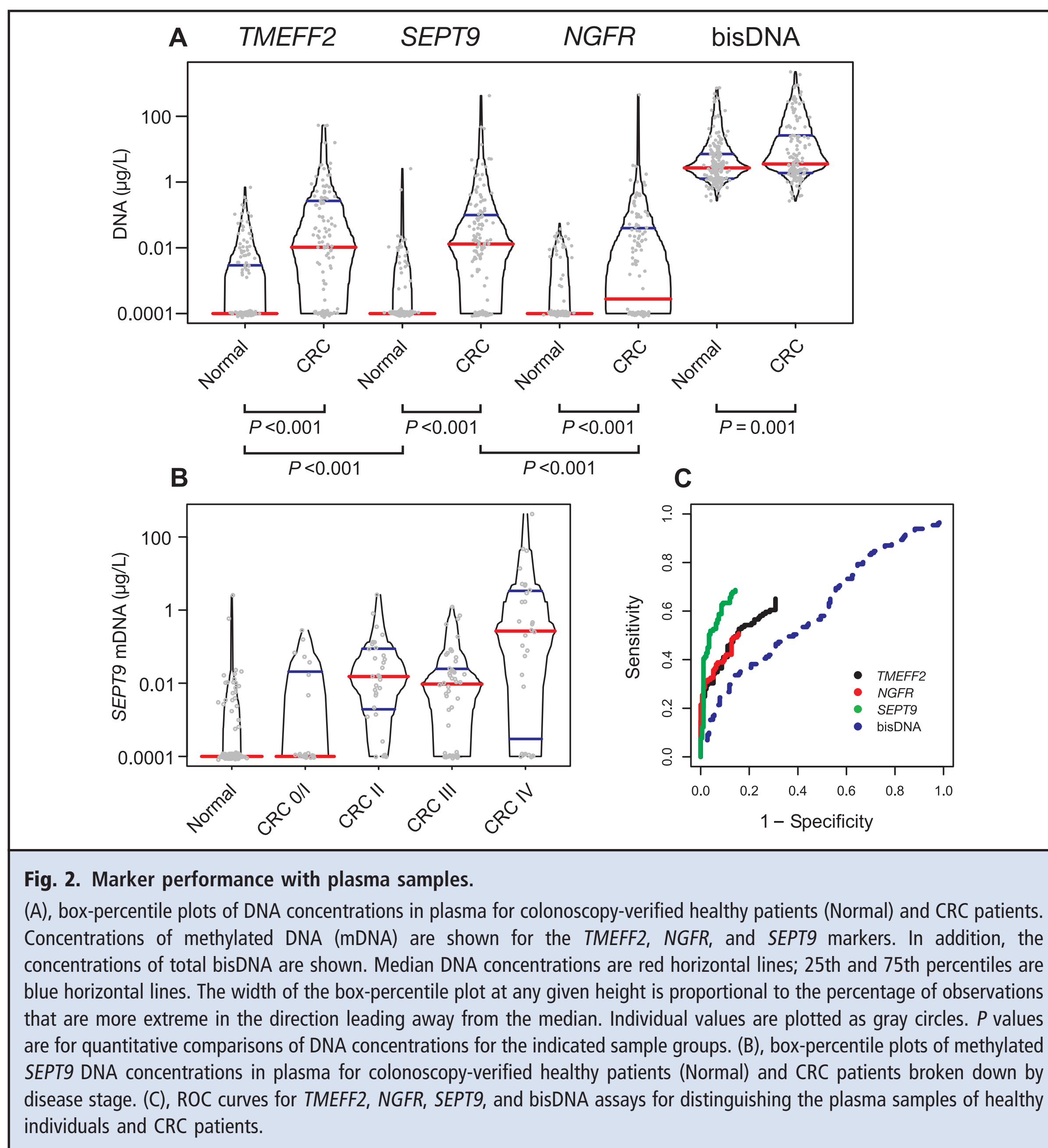

Fig. 2. Marker performance with plasma samples.

(A), box-percentile plots of DNA concentrations in plasma for colonoscopy-verified healthy patients (Normal) and CRC patients. Concentrations of methylated DNA (mDNA) are shown for the *TMEFF2*, *NGFR*, and *SEPT9* markers. In addition, the concentrations of total bisDNA are shown. Median DNA concentrations are red horizontal lines; 25th and 75th percentiles are blue horizontal lines. The width of the box-percentile plot at any given height is proportional to the percentage of observations that are more extreme in the direction leading away from the median. Individual values are plotted as gray circles. *P* values are for quantitative comparisons of DNA concentrations for the indicated sample groups. (B), box-percentile plots of methylated *SEPT9* DNA concentrations in plasma for colonoscopy-verified healthy patients (Normal) and CRC patients broken down by disease stage. (C), ROC curves for *TMEFF2*, *NGFR*, *SEPT9*, and bisDNA assays for distinguishing the plasma samples of healthy individuals and CRC patients.

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little complementarity (Fig. 1B). Therefore, the improvement in discrimination performance over the best single marker was small (Table 2). The best-performing marker panel was the combination of *SEPT9* and *NGFR*, with a slight but significant bias-corrected AUC improvement of 2% over *SEPT9* alone ($P = 0.03$).

VALIDATION OF BIOMARKER ASSAYS WITH PLASMA SAMPLES

Final assay optimization. The detection of methylated target in plasma requires assays that amplify extremely low quantities of methylated tumor-marker DNA in a vast excess of unmethylated or partially methylated background DNA. To achieve this combination of technical sensitivity and specificity, we optimized qPCR assays selected from the tissue studies that used the HM technology [*TUSC3*, tumor suppressor candidate 3; *TMEFF2*; *ZDHHC22*, zinc finger, DHHC-type containing 22; *VTN*; *FOXL2*, forkhead box L2; *NGFR*; *ALX4*, aristaless-like homeobox 4; *SIX6*, SIX homeobox 6; *EYA4*; and *SEPT9*] *(14)*. The HM assays

Table 3. Plasma performance of single markers.

	TMEFF2	*NGFR*	*SEPT9*	bisDNA
Assay LOD in background of 50 ng PBL DNA, pg/reaction	70	21	19	
Positives, %				
Normal plasma[a]	31	16	14	100
CRC plasma	65	51	69	100
Median DNA (90% range), mg/L				
Normal plasma	0 (0–0.078)	0 (0–0.017)	0 (0–0.010)	2.7 (0.7–112.3)
CRC plasma	0.010 (0–3.316)	0 (0–0.937)	0.013 (0–4.105)	3.5 (0.6–498.4)
AUC	0.72	0.70	0.80	0.61
P	0	0	0	0.0001
Cutoff for 95% specificity for normal plasma (n = 179), mg/L	0.098	0.019	0.011	150.3
Sensitivity at 95% specificity by CRC stage, %				
All stages (n = 133)[b]	30	33	52	15
Stage 0/I (n = 20)	5	20	30	0
Stage II (n = 32)	22	25	56	16
Stage III (n = 47)	34	36	45	21
Stage IV (n = 31)	45	36	68	16

[a] Normal plasma indicates plasma from healthy individuals (i.e., non-CRC).
[b] Includes 3 CRC patient samples of unknown stage.

were required to meet several criteria. First, the absolute technical sensitivity of the assay had to be a maximum of 25 pg. Second, the assay could show no amplification of 50 ng of unmethylated DNA. Third, the assay had to detect at least 25 pg of methylated DNA in a background of 50 ng of unmethylated DNA. The correlation between the initial MethyLight assays and the HM versions was required to be >0.8 with a subset of previously tested tissue samples. The LOD in blood was required to be, at minimum, 50 pg of methylated DNA in a background of 50 ng PBL DNA, tested on several batches. Lastly, the positive rate in plasma from healthy individuals, measured as the proportion of healthy plasma samples showing amplification, should not be >20%.

The first 3 of these criteria are stricter versions of the analytical-performance requirements determined for tissue-based studies. The correlation criterion was introduced to ensure that the final assay measures the same biological information as the original candidate assay. The LOD criterion ensures that the assays reliably detect methylated target molecules in blood. The final filter requires reasonable specificity for a small number of plasma samples and ensures the healthy tissue–based and PBL-based model system did not miss any dominant source of methylated target DNA.

We eliminated 1 HM assay (*ZDHHC22*) during analytical testing because of a lack of concordance with MethyLight results. Two HM assays failed to reliably detect methylated targets in a blood background because of a low level of methylation in some PBL batches (*TMEFF2* LOD, 70 pg; *TUSC3* LOD, >100 pg). Although the results were negative with human PBLs, the *VTN* assay was eliminated after it showed high concentrations of methylated target in healthy human plasma.

Study design and results of the control assay. Because of the limited volume of plasma sample per patient, only 3 HM assays could be tested with plasma. We selected *SEPT9* because of its superior performance with respect to all of the selection criteria. *NGFR* was selected despite its low methylation rates in CRC tissue samples because it showed the highest complementarity to *SEPT9* in the multivariate tissue analysis. Despite its failure to meet our LOD criterion, we selected *TMEFF2* to compare our results with the existing literature *(7, 21)* and to test our hypothesis that the degree of PBL amplification predicts plasma specificity.

The set of plasma samples included 320 samples from 185 healthy individuals (median age, 56 years; 60% female) and 135 patients with CRC (median age, 65 years; 50% female). Six samples from the healthy category were omitted from analysis (3 because of no measurable bisDNA and 3 because of sample-processing errors), and 2 samples from the CRC category were omitted from analysis (1 because of no measurable

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bisDNA and 1 because of radiotherapy before blood draw). The results showed a significantly higher median bisDNA amount in plasma samples from CRC patients than in plasma samples from healthy individuals ($P < 0.001$). The distributions in DNA concentration for the 2 classes showed very strong overlap (Fig. 2A), however, and class differentiation was weak (AUC, 0.61) (Table 3).

Clinical performance of methylation markers. A qualitative analysis indicated the most sensitive marker to be *SEPT9*, which amplified 69% of CRC plasma samples, followed by *TMEFF2* (65%) and *NGFR* (51%) (Table 3). *NGFR* and *SEPT9* had similar specificities and amplified 16% and 14% of plasma samples from healthy individuals, respectively. The least specific marker was *TMEFF2*, with 31% positivity for plasma samples from healthy individuals. Consequently, *SEPT9* predicted the presence of CRC significantly more accurately than *TMEFF2* ($P < 0.001$) or *NGFR* ($P < 0.01$). There was no significant difference in diagnostic accuracy between the *TMEFF2* and *NGFR* markers.

A quantitative analysis demonstrated *SEPT9* to have significantly higher concentrations of methylated DNA in plasma samples from CRC patients than *NGFR* ($P < 0.001$, Fig. 2A). In plasma samples from healthy individuals, *SEPT9* had significantly lower concentrations of methylated DNA than *TMEFF2* ($P < 0.001$). *TMEFF2*, *SEPT9*, and bisDNA showed a significant increase in target DNA to be correlated with the stage of disease ($P < 0.05$, Fig. 2B); *NGFR* showed no significant difference between disease stages. Consequently, the biomarker with the highest performance was *SEPT9* (AUC, 0.80; Fig. 2C). A cutoff of 0.011 μg/L of methylated *SEPT9* DNA produces a specificity of 95% and a sensitivity of 52%.

We performed multivariate logistic regression analyses for all combinations of these 3 markers (Table 2). All 3 markers were highly correlated for a subset of plasma samples from CRC patients with high concentrations of tumor DNA (Fig. 3). For cancer samples with amounts of tumor DNA close to or below the LOD, the amplification results were in the stochastic range, and the markers were no longer correlated. As with the analysis of tissue samples, the best performing marker panel was the combination of *SEPT9* and *NGFR*, with a small but significant bias-corrected AUC improvement of 1% over *SEPT9* alone ($P = 0.04$) (Table 2).

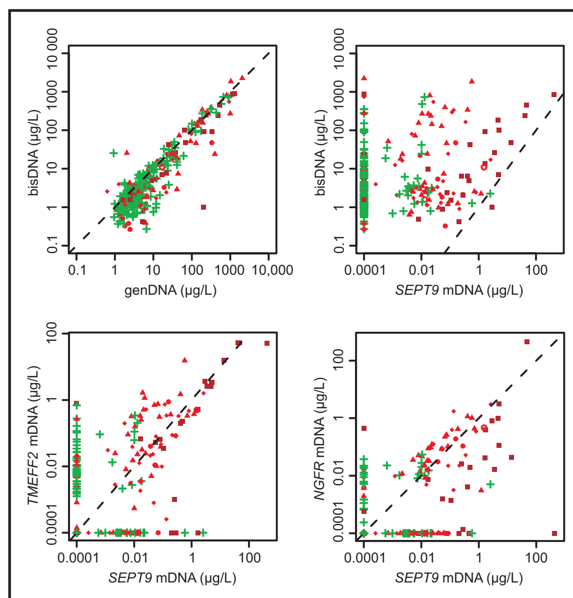

Fig. 3. Correlations of markers for plasma samples.
Pair-wise correlations between concentrations of total genomic DNA (genDNA), total bisDNA, and methylated DNA from *SEPT9*, *TMEFF2*, and *NGFR* in plasma samples from a set of colonoscopy-verified healthy patients (green crosses) and CRC patients (red points, stage 0/I; red diamonds, stage II; red triangles, stage III; red squares, stage IV; red open circles, unknown stage). genDNA concentrations were on average 1.35-fold higher than total bisDNA concentrations, indicating a minimal loss of DNA with bisulfite treatment.

Discussion

A variety of genome-wide methods are currently available for the discovery of differentially methylated markers *(22, 23)*. These methods typically produce large numbers of potential candidates; thus, downstream selection processes are critical for identifying clinically relevant markers that have the properties necessary to perform adequately in future tests. To ensure adequate sensitivity and specificity in plasma samples, we have experimentally established benchmarks for the performance of analytical assays, for minimum methylation levels in CRC tissues, and for maximum tolerable methylation levels in PBLs (see Table 4 in the online Data Supplement for an overview). For example, the qPCR assay for *TMEFF2*, although fairly specific with PBLs from healthy individuals, does not provide the level of specificity needed for use as a screening test without first establishing a cutoff concentration that consequently reduces the marker's sensitivity. Our results with plasma are somewhat contradictory to those of Sabbioni et al., who found a 100% specificity for the 16 healthy control individuals they tested *(7)*. We suspect that methylation of *TMEFF2* occurs randomly in PBLs in the healthy population, although at a very low incidence, making this marker more appropriate for applications that do not have high specificity requirements. On the other hand, the *NGFR* and

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SEPT9 assays have equally good PBL specificities and analytical sensitivities, but the *SEPT9* assay detects much higher levels of methylation in individual samples of CRC tissue, a phenomenon that appears to manifest as a higher clinical sensitivity in plasma samples. It is this type of marker assay that has the greatest chance for success as a screening marker in blood, and efforts are currently focused on the commercial development of this marker for the early detection of CRC. We found several other markers that passed all stages of marker validation but were not included in the final plasma testing because of insufficient sample volumes. These markers (*ALX4*, *EYA4*, *FOXL2*, and *SIX6*) will be tested in subsequent studies to evaluate their clinical performance.

Patient compliance and the performance of current noninvasive screening strategies limit the effectiveness of CRC screening tests available on the market today. Because of the relative ease of performing blood-screening evaluations, tests of this type can be administered regularly during routine physical examinations. An easily administered blood-based test for the early detection of CRC followed by colonoscopy for individuals with positive results has the potential to be an effective tool for reducing mortality from this disease. This study has developed a discovery/validation process that produces promising candidates for use in screening tests and other potential diagnostic applications. Further validation is now focused on testing the markers, particularly *SEPT9*, in larger studies that include plasma samples from CRC patients, individuals with preneoplastic disease, and other healthy and disease controls. Application as a screening biomarker will require a large prospective trial in an asymptomatic, CRC-screening guideline– eligible population.

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