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Spectrin Repeat Containing Nuclear Envelope 1 and Forkhead Box Protein E1 Are Promising Markers for the Detection of Colorectal Cancer in Blood

Veerle Melotte¹, Joo Mi Yi², Marjolein H.F.M. Lentjes¹, Kim M. Smits^{1,3}, Leander Van Neste^{1,4}, Hanneke E.C. Niessen¹, Kim A.D. Wouters¹, Joost Louwagie⁴, Kornel E. Schuebel⁵, James G. Herman⁵, Stephen B. Baylin⁵, Wim van Criekinge⁴, Gerrit A. Meijer⁶, Nita Ahuja^{5,7,8}, and Manon van Engeland¹

¹Department of Pathology, GROW-School for Oncology and Developmental Biology, Maastricht University Medical Center, Maastricht, the Netherlands. ²Dongnam Institute of Radiological and Medical Sciences (DIRAMS), Busan, South Korea. ³Department of Radiation Oncology (MAASTRO), GROW-School for Oncology and Developmental Biology, Maastricht University Medical Center, Maastricht, the Netherlands. ⁴MDxHealth, Herstal, Belgium. ⁵Department of Oncology, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins University School of Medicine, Baltimore, Maryland. ⁶Department of Pathology, VU University Medical Center, Amsterdam, the Netherlands. ⁷Department of Surgery, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins University School of Medicine, Baltimore, Maryland. ⁸Department of Urology, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins University School of Medicine, Baltimore, Maryland.

Abstract

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Corresponding Authors: Manon van Engeland, Department of Pathology, GROW-School for Oncology and Developmental Biology, Maastricht University Medical Center, P.O. Box 5800, Maastricht, the Netherlands. Phone: 31-614030164; Fax: 31-433876613; manon.van.engeland@mumc.nl; and Nita Ahuja, nahuja@jhmi.edu.

V. Melotte, J.M. Yi, N. Ahuja, and M. van Engeland contributed equally to this article.

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Authors' Contributions

Conception and design: V. Melotte, J.M. Yi, N. Ahuja, M. van Engeland

Development of methodology: K.M. Smits, K.E. Schuebel, J.G. Herman, W. van Criekinge, N. Ahuja, M. van Engeland

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H.E.C. Niessen, K.A.D. Wouters, J. Louwagie, N. Ahuja, M. van Engeland

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V. Melotte, M.H.F.M. Lentjes, K.M. Smits, L. Van Neste, W. van Criekinge, N. Ahuja, M. van Engeland

Writing, review, and/or revision of the manuscript: V. Melotte, M.H.F.M. Lentjes, K.M. Smits, L. Van Neste, J.G. Herman, W. van Criekinge, G.A. Meijer, N. Ahuja, M. van Engeland

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.H.F.M. Lentjes, K.M. Smits, K.A.D. Wouters, S.B. Baylin, G.A. Meijer, N. Ahuja

Study supervision: N. Ahuja, M. van Engeland

Identifying biomarkers in body fluids may improve the noninvasive detection of colorectal cancer. Previously, we identified *N-Myc downstream-regulated gene 4 (NDRG4)* and *GATA binding protein 5 (GATA5)* methylation as promising biomarkers for colorectal cancer in stool DNA. Here, we examined the utility of *NDRG4*, *GATA5*, and two additional markers [*Forkhead box protein E1 (FOXE1)* and *spectrin repeat containing nuclear envelope 1 (SYNE1)*] promoter methylation as biomarkers in plasma DNA. Quantitative methylation-specific PCR was performed on plasma DNA from 220 patients with colorectal cancer and 684 noncancer controls, divided in a training set and a test set. Receiver operating characteristic analysis was performed to measure the area under the curve of *GATA5*, *NDRG4*, *SYNE1*, and *FOXE1* methylation. Functional assays were performed in *SYNE1* and *FOXE1* stably transfected cell lines. The sensitivity of *NDRG4*, *GATA5*, *FOXE1*, and *SYNE1* methylation in all stages of colorectal cancer (154 cases, 444 controls) was 27% [95% confidence interval (CI), 20%–34%), 18% (95% CI, 12%–24%), 46% (95% CI, 38%–54%), and 47% (95% CI, 39%–55%), with a specificity of 95% (95% CI, 93%–97%), 99% (95% CI, 98%–100%), 93% (95% CI, 91%–95%), and 96% (95% CI, 94%–98%), respectively. Combining *SYNE1* and *FOXE1*, increased the sensitivity to 56% (95% CI, 48%–64%), while the specificity decreased to 90% (95% CI, 87%–93%) in the training set and to 58% sensitivity (95% CI, 46%–70%) and 91% specificity (95% CI, 80%–100%) in a test set (66 cases, 240 controls). *SYNE1* overexpression showed no major differences in cell proliferation, migration, and invasion compared with controls. Overexpression of *FOXE1* significantly decreased the number of colonies in SW480 and HCT116 cell lines. Overall, our data suggest that *SYNE1* and *FOXE1* are promising markers for colorectal cancer detection.

Introduction

Early detection of colorectal cancer offers opportunity to cure colorectal cancer (1). Colonoscopy is the gold standard for colorectal cancer screening, however this procedure is invasive, expensive, and not readily accessible or acceptable to the majority of age-eligible individuals (2). Therefore, the search for noninvasive screening methods has intensified. Currently, fecal immunochemical test (FIT) is used as a noninvasive and relatively inexpensive colorectal cancer screening modality. In addition, potential biomarkers in stool DNA have been described. Despite the high sensitivity and specificity of some of these markers in stool DNA, we hypothesized that a blood-based test, not depending on stool sampling, has the potential for better patient compliance and is better suited for systems without programmatic screening.

Epigenetic silencing of tumor-suppressor genes by aberrant promoter methylation frequently occurs in human cancers (3). Promoter methylation is suggested to be an early event in carcinogenesis and can be detected in biologic fluids in various cancers (4–7). Body fluids that have been used for cancer screening with methylation markers include urine (8), ejaculates of men (9), salivary rinses (10), sputum (11), peritoneal fluid (12), and ductal lavage and nipple fluid (13, 14) highlighting the potential for application in routine clinical practice.

For colorectal cancer, we and others have shown that detection of promoter methylation in fecal DNA holds promise as a colorectal cancer prescreening modality (7, 15–20). Genes

known to be methylated, detected, and studied in tumor-derived DNA in blood of patients with colorectal cancer are *ALX4* (21), *TMEFF2* (22), *CDKN2A* (*p16*; ref. 23), *CDH4* (24), *NEUROG1* (25), *NGFR* (22), *SEPT9* (22), *MLH1* (26, 27), *DAPK* (28), *THBD* (29), *SDC2* (30), and gene panels consisting of *APC*, *MLH1*, and *HLTF* (27), and *APC*, *MGMT*, *RASSF2A*, and *Wif-1* (31). The sensitivity and specificity to detect colorectal cancer observed in these studies range from 21% to 86% and from 69% to 100%, respectively.

Our objective was to examine promoter methylation of two previously identified stool markers (*NDRG4* and *GATA5*; refs. 15, 18) and two novel markers namely *FOXE1* and *SYNE1* (32), as potential biomarkers for the early detection of colorectal cancer in blood DNA. *FOXE1* and *SYNE1* were identified as frequently methylated genes using a transcriptome-wide approach to identify genes that are transcriptionally silenced by methylation in colorectal cancer (32). In addition, methylation of *SYNE1* and *FOXE1* has been described in a small cohort of patients with colitis-associated colorectal neoplasia (33).

Performance of the best combinatorial marker panel was evaluated by quantitative methylation analysis in two large sets of plasma samples from patients with colorectal cancer and controls. Furthermore, the currently unknown functional role of *SYNE1* and *FOXE1* in colorectal cancer was investigated.

Materials and Methods

Study population plasma samples

Two hundred and twenty plasma samples were prospectively collected from patients with colorectal cancer from multiple centers in Germany. Symptomatic patients were screened using colonoscopy and the clinical diagnosis of colorectal cancer was confirmed by histology. The trial started in 2007 and recruited patients with all disease stages. Included patients were diagnosed with colorectal cancer, had not been treated for colorectal cancer before blood collection, had not been treated for other malignancies during the previous 5 years and had surgery planned to assess the UICC stage and the involvement of lymph nodes.

Control blood samples ($n = 664$) were collected from 550 asymptomatic average risk and 134 symptomatic individuals, all without adenomas and/or colorectal cancer detected by colonoscopy screening. These individuals were enrolled in a multicenter colorectal cancer screening trial in Germany of average risk subjects. Participants underwent primary colonoscopy screening and blood samples were drawn before the procedure. Patient characteristics of patients with colorectal cancer and controls are shown in Supplementary Table S1. Plasma samples of patients with colorectal cancer and controls were randomized and divided in two different sets, one training and one test set, as depicted in Supplementary Fig. S1. Informed consent was obtained from all participants, adhering to ethics guidelines.

Collection and isolation of plasma samples

Nine milliliters of blood, using 10 mL EDTA Vacutainer tubes (BD Vacutainer; BD Hemogard, K2 EDTA spray-dried; cat no. 367525), was collected using standard venipuncture techniques. Plasma was separated by centrifugation at $1,500 \times g$ for 15 minutes

(double spin) within 2 hours of collection. DNA isolation from plasma was performed using the QIAamp Circulating Nucleic Acid Test Kit (Qiagen; cat no. 55114).

Sodium bisulfite treatment and quantitative methylation-specific PCR

Sodium bisulfite modification was performed using the EpiTect Bisulphite Kit (Qiagen; cat no. 59104) according to the manufacturer's instructions. Quantitative methylation-specific PCR on plasma samples was performed on a 7900HT real-time PCR system (Applied Biosystems). For the training set, two duplex PCRs *NDRG4_FAM/FOXE1_TET* and *GATA5_FAM/SYNE1_TET* were performed. Based on the performance of the first duplex reaction, only *SYNE1* and *FOXE1* were retained in the test set (one duplex PCR; *SYNE1_TET/FOXE1_HEX*). The PCR master mix was 30 μ L QuantiTect Multiplex PCR with ROX dye (Qiagen), 10 μ L template volume, forward primer (0.28 μ mol/L) of both genes, reverse primer (0.28 μ mol/L) of both genes and a single-stranded oligonucleotide hybridization probe (0.25 μ mol/L) of both genes. The PCR program was: 15 minutes at 95°C; followed by 45 cycles of 30 seconds at 95°C, 30 seconds at 57°C, and 30 seconds at 72°C; followed by 5 minutes at 72°C. Serially diluted plasmids containing the target sequence were amplified to generate a standard curve against which the unknown samples are quantified by interpolation of their PCR cycle number (C_t value) to the corresponding plasmid copy. Because the yield showed very high intersubject variability, no direct control for DNA yield was incorporated. This standard curve ruled out any technical or reagent batch-related errors in methylation calls. Samples were handled and analyzed in a blinded fashion during storage, DNA isolation, and PCR analysis. Primer and probe sequences are provided in Supplementary Table S2.

Cell culture and transfections

Human colorectal cancer cell lines were cultured in Dulbecco's MEM (DMEM; Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FBS; HyClone). Cell lines (RKO, SW480, and HCT116) were purchased from ATCC (LGC standards). No authentication was done by the authors. Full-length *FOXE1* cDNA was subcloned into the pIRES-neo3 expression vector (Clontech Laboratories Inc.). GFP-*SYNE1* and GFP-*SYNE1*-KASH (Klarischt) were kindly provided by Dr. Zhang (Department of Medicine, Cambridge, United Kingdom).

HCT116 cells were transfected with the Nucleofector Kit V (Amaxa Biosystems) and RKO and SW480 cells were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. HCT116, RKO, and SW480 cells were transfected with a control construct (empty vector) or *FOXE1*-pIRESneo3, GFP-*SYNE1*, and GFP-*SYNE1*-KASH, selected for 10 days with G418 (HCT116 and SW480 400 μ g/mL; RKO 1 mg/mL).

Colony formation assay

RKO, HCT116, and SW480 CRC cells were transfected in 6-well plates with control construct *FOXE1*-pIRESneo3, GFP-*SYNE1*, or GFP-*SYNE1*-KASH as described above. The next day, cells were diluted 1:20 and G418 (RKO 1 mg/mL; HCT116, SW480 400 μ g/mL) was added. After 14 days of selection, colonies were stained and quantified.

***In vitro* cell proliferation, migration, and invasion assays**

HCT116, RKO, and SW480 cells were seeded onto 96-well plates (5,000 cells per well) and after 96 hours, the cultures were pulsed for 6 hours with 0.3 μCi [methyl-3H] thymidine (Amersham Life Science) per well. Activity was measured using liquid scintillation. Cell migration and invasion assays were performed using 24-well Transwells (8- μm pore size) coated with (invasion) or without (migration) Matrigel (BD Biosciences). HCT116, RKO, and SW480 cells (20×10^4) in 1% FBS-DMEM were seeded into the upper chamber, and DMEM containing 20% FBS was placed in the lower chamber. After 48 hours, cells on the lower surface of the membrane were fixed with methanol and stained with 1% Toluene Blue in 1% borax.

Data analysis

For quantitative methylation-specific PCR analysis, receiver operating characteristics (ROC) curve analysis and the area under the curve (AUC) were determined to define the best markers with highest sensitivity and specificity. *GATA5*, *NDRG4*, *SYNE1*, and *FOXE1* promoter methylation was considered positive if copies of any of these genes were detected. The measured methylated copy numbers were used as the basis for the age- and gender-association analyses, hence methylation was implemented as a continuous variable. Age was stratified in two groups, i.e., those patients younger than 65 and those age 65 and above. The Mann–Whitney rank-sum test was used to analyze the colony formation, migration, and invasion assays.

Results

***NDRG4*, *GATA5*, *FOXE1*, and *SYNE1* methylation in blood DNA as a potential biomarker for colorectal cancer detection**

The main objective of this study was to investigate the use of *NDRG4*, *GATA5*, *SYNE1*, and *FOXE1* promoter methylation as biomarkers for detection of colorectal cancer–derived DNA in plasma by determining the optimal classifier in a training set and subsequently confirming this in a test set.

Training set

Using 444 normal control samples and 154 colorectal cancer samples of the training set, we evaluated *NDRG4*, *GATA5*, *SYNE1*, and *FOXE1* as single marker candidates using ROC curve analysis. The AUC generated for plasma *NDRG4*, *GATA5*, *SYNE1*, and *FOXE1* methylation was 0.61 (95% CI, 0.57–0.65; Fig. 1A), 0.59 (95% CI, 0.55–0.63; Fig. 1B), 0.72 (95% CI, 0.68–0.75; Fig. 1C), and 0.70 (95% CI, 0.69–0.73; Fig. 1D), respectively, indicating that *SYNE1* and *FOXE1* have the highest performance as potential biomarkers.

To study the sensitivity and specificity of these markers separately, we defined a cutoff of zero. The sensitivity of *NDRG4* methylation in all stages of colorectal cancer was 27% (95% CI, 20%–34%) with a specificity of 95% (95% CI, 93%–97%). The overall sensitivity of *GATA5* methylation was 18% (95% CI, 12%–24%) with a specificity of 99% (95% CI, 98%–100%). *FOXE1* methylation has a sensitivity of 46% (95% CI, 38%–54%) in detecting patients with colorectal cancer and a specificity of 93% (95% CI, 91%–95%). Finally,

SYNE1 methylation generated a sensitivity of 47% (95% CI, 39%–55%) for detection of colorectal cancer and a specificity of 96% (95% CI, 94%–98%). Early-stage colorectal cancers were detected at slightly lower rates than later stages, a phenomenon that occurred for three of the four markers analyzed (except *SYNE1*), as depicted in Table 1.

The presence of methylated *NDRG4* ($P = 0.45$, $P = 0.12$), *GATA5* ($P = 0.60$; $P = 0.13$), *FOXE1* ($P = 0.88$; $P = 0.053$), and *SYNE1* ($P = 0.39$; $P = 0.15$) in plasma was not associated with age and gender, respectively. Given the low sensitivity of *GATA5* and *NDRG4* methylation, these markers did not add to the overall sensitivity (data not shown). In addition, combining *SYNE1* and *FOXE1* methylation increased the sensitivity to 56% (95% CI, 48%–64%) with a specificity of 90% (95% CI, 87%–93%; Table 2).

Test set

To confirm the clinical performance of *SYNE1* and *FOXE1* methylation as a multimarker panel, we tested plasma samples obtained from 66 patients with colorectal cancer and 240 healthy controls. Using the same threshold, we observed a sensitivity of 58% (95% CI, 46%–70%) and a specificity of 91% (95% CI, 80%–100%) for all stages of colorectal cancer for *FOXE1* and *SYNE1*, respectively (Table 3). Stage I [37% (95% CI, 19%–55%)] and stage III [55% (95% CI, 33%–77%)] cancers were detected at lower rates than stage II [87% (95% CI, 70%–100%)], and stage IV [100% (95% CI, NA)] cancers (Table 3). *SYNE1* and *FOXE1* promoter methylation was not associated with age (*FOXE1*, $P = 0.36$ and *SYNE1*, $P = 0.38$) and gender (*FOXE1* $P = 0.29$ and *SYNE1* $P = 0.28$).

FOXE1 and SYNE1 overexpression in colorectal cancer cell lines

The functional role of *NDRG4* and *GATA5* *in vitro* in colorectal cancer cell lines has been previously described (15, 18). Currently, nothing is known about the role of *SYNE1* and *FOXE1* in colorectal cancer.

To examine whether *SYNE1* has tumor-suppressor activities in colorectal cancer cells, HCT116 and RKO cells were transfected with *SYNE1* expression constructs. Transfection with GFP-*SYNE1* and GFP-*SYNE1*-KASH induced the number of G418-resistant colonies as compared with control transfectants in HCT116 (Fig. 2A, $P = 0.001$ and $P = 0.07$) and RKO (Fig. 2B, $P = 0.13$ and $P = 0.17$) cells. Migration (Fig. 2C; HCT116, $P = 0.26$ and $P = 0.16$ and 2D; RKO, $P = 0.19$ and $P = 0.07$) and invasion (Fig. 2E; HCT116, $P = 0.29$, and $P = 0.20$) and 2F; RKO, $P = 0.27$ and $P = 0.03$) of *SYNE1*-transfected cells was higher than that of control clones. However, although a trend was seen in all cell lines, most results were not statistically significant.

To investigate whether *FOXE1* is a putative tumor suppressor in colorectal cancer, we first performed *in vitro* colony formation assays to determine the effects of full-length *FOXE1* transfected into SW480, HCT116, and RKO cells. Overexpression with *FOXE1* resulted in a significant reduction of G418-resistant colonies in HCT116 and SW480 cells [a nearly 2-fold reduction in HCT116 (Fig. 3A, $P = 0.003$) and more than 8-fold reduction in SW480 cells (Fig. 3C, $P = 0.006$)]. No significant effect was observed in RKO cells transfected with *FOXE1* (Fig. 3B, $P = 0.577$). Overexpression of the *FOXE1* gene had no significant effect

on the migration [Fig. 3D (HCT116, $P = 0.63$), E (RKO, $P = 0.82$), F (SW480, $P = 0.28$)] and invasion [Fig. 3G HCT116, $P = 0.79$), H (RKO, $P = 0.92$), I (SW480, $P = 0.38$)] of these cells. Taken together, these data suggest that *FOXE1* may have tumor-suppressive effects in human colorectal cancer cells.

Discussion

We previously published *NDRG4* (18) and *GATA4/GATA5* (15) promoter hypermethylation as potential sensitive and specific stool DNA markers and candidate tumor-suppressor genes in colorectal cancer. The potential of *NDRG4* methylation for the detection of colorectal cancer was independently confirmed by Alquist and colleagues and Imperiale and colleagues, who recently developed a multimarker diagnostic DNA test, including *NDRG4* methylation, to screen for colorectal cancer (19, 34). This DNA test has shown to perform better compared with analyzing FIT alone. However, implementation of this test might be hampered in population-based screening programs due to the requirement of a complete stool sample affecting compliance and cost-effectiveness. Despite the high potential of these markers in stool DNA, we hypothesized that a blood-based test for colorectal cancer detection, not depending on stool sampling, has the potential for better patient compliance and is better suited for systems without programmatic screening. Therefore, we investigated the performance of our previously reported stool methylation markers (*NDRG4* and *GATA5*) and two other promising methylation markers (*SYNE1* and *FOXE1*) in blood. *FOXE1* and *SYNE1* were identified as frequently methylated genes in a transcriptome-wide approach to detect transcriptionally silenced genes by promoter CpG island methylation in colorectal cancer (32).

FOXE1 is a transcription factor that is characterized by a distinct forkhead domain, and plays a crucial role in thyroid morphogenesis. In thyroid cancer, *FOXE1* is upregulated (35), although most cancers have a decreased expression of *FOXE1* often due to promoter CpG island methylation as has been described in breast cancer (36), pancreatic cancer (37), and in cutaneous squamous cell carcinoma (38). Furthermore, *FOXE1* has been reported as a sensitive (82%) and specific (98.5%) methylation marker for pancreatic cancer in pancreatic juice of patients (37).

We and others described DNA methylation of *SYNE1* in colorectal cancer, suggesting a tumor-suppressor function in colorectal cancer (32, 39). *SYNE1* promoter methylation and a possible tumor-suppressor function have been reported in lung cancer (40). However, the *in vitro* studies in this study show that *SYNE1* overexpression in colorectal cancer cell lines induces cell proliferation, migration, and invasion indicating an oncogenic role for *SYNE1* in colorectal cancer. Interestingly, in line with our functional data, gene expression data obtained from GEO from primary normal colon and colon cancer showed no decrease of *SYNE1* expression in colorectal cancer tissues (data not shown), speculating that promoter methylation of *SYNE1* in colorectal cancer does not result in downregulation of this gene.

We have studied the role of *NDRG4* in colorectal cancer and found that *NDRG4* significantly inhibited colony formation, cell proliferation, and invasion, and is frequently methylated (70% and 86% in two independent series) in colorectal cancer tissue samples

(18). In parallel, we have published the potential tumor-suppressor role of GATA5 in colorectal cancer, which inhibited colony formation, cell growth, migration, invasion, and anchor-age-independent growth *in vitro*. Furthermore, promoter methylation of GATA5 was observed (79% and 74% in two independent series) in colorectal cancer tissues (15). GATA5 promoter methylation has been reported in other tumor types as well, such as small-cell lung cancer, pancreatic, esophageal, ovarian, and gastric cancer (41–45). In the present article, we investigated the performance of *NDRG4*, *GATA5*, *SYNE1*, and *FOXE1* as methylation markers in blood DNA. Interestingly, although *NDRG4* performed well as a methylation marker in stool (sensitivity of 61% and 53% for the training and test set, respectively), the detection rate in blood for colorectal cancer was only 27%. Molecular pathways and cellular mechanisms that underlie multistage processes of metastasis, including tumor invasion, tumor-cell dissemination through the bloodstream or the lymphatic system, colonization of distant organs and, outgrowth of metastases have been characterized (46). A difference in gene expression at the invasive front compared with the central area of the tumor and the luminal part of the tumor has been described for beta-catenin (47). This heterogeneity in gene expression probably indicates heterogeneity in the underlying mechanisms that are regulating gene expression. We therefore hypothesize that molecular or morphologic characteristics at the invasive front area of colorectal cancer, a part of the tumor that probably also easily invades blood vessels, may be different from the luminal part of the colorectal cancer that is shedding tumor cells in stool. An alternative explanation for differences in sensitivity in blood or fecal DNA could be the DNA isolation procedure. Diehl and colleagues demonstrated that the majority of mutated *APC* sequences in patients with colorectal cancer were detected in smaller size fragments of circulating DNA, whereas larger fragments tended to be wild-type (48). Therefore, improvement of isolation protocols of blood DNA, could, in theory, yield a blood DNA test for *NDRG4* with higher sensitivity.

We here describe a biomarker panel of two genes able to discriminate colorectal cancer from non-colorectal cancer in plasma with a sensitivity of 56% and 58% and a specificity of 90% and 91%. Of all blood-based DNA markers, plasma septin-9 has been studied most extensively. Test sensitivity varies widely for both colorectal cancer (from 48% to 90%) and adenomas (from 11% to 29%), whereas specificity was more consistent ranging from 86% to 97% (49). Septin-9 was also evaluated in a screening setting, where sensitivity and specificity were 50% and 91%, respectively, for patients with colorectal cancer when two aliquots were tested and 64% and 88%, respectively, with three samples tested (49). Further research is necessary to investigate whether the sensitivity of *SYNE1* and *FOXE1* can be improved using different aliquots and whether it will be effective and cost-effective compared with no screening and compared with other screening test available. In addition, these data need to be validated in a large prospective colorectal cancer screening study and should be combined with other noninvasive screening test such as genetic or epigenetic DNA markers or the FIT to enhance sensitivity. Furthermore, sample collection, DNA isolation, bisulfite conversion, and assay sensitivity should be optimized. Detecting minute amounts of tumor DNA in blood, especially in the early-stage tumors, and defining the specificity of a DNA methylation marker in blood that contain DNA from many sources in the body are challenges for future research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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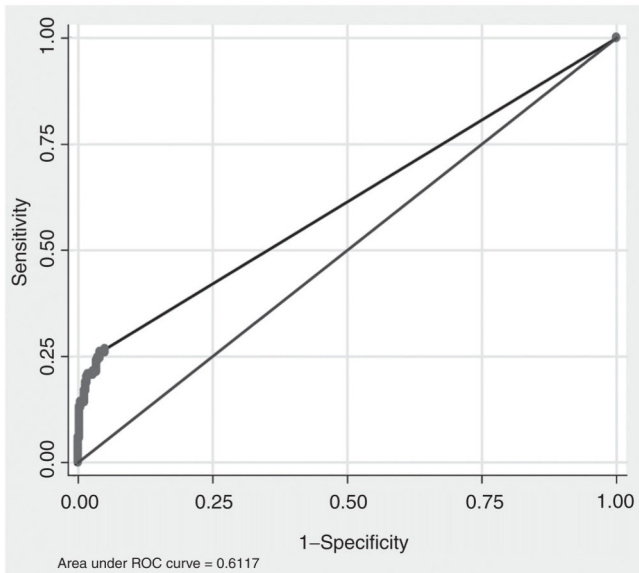
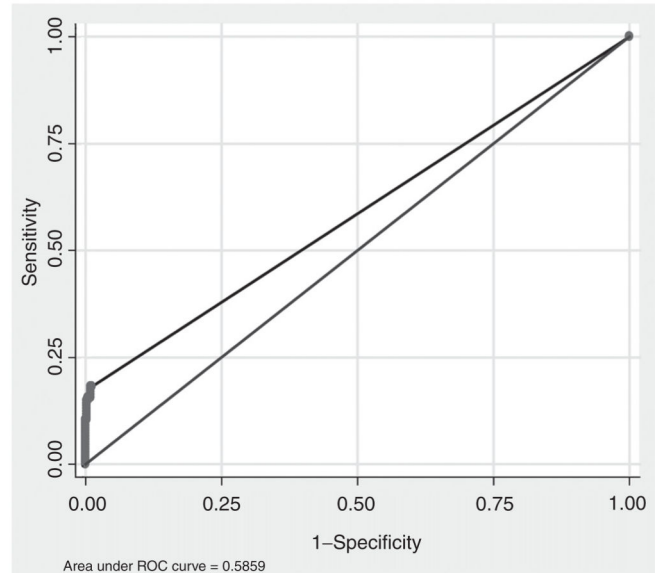
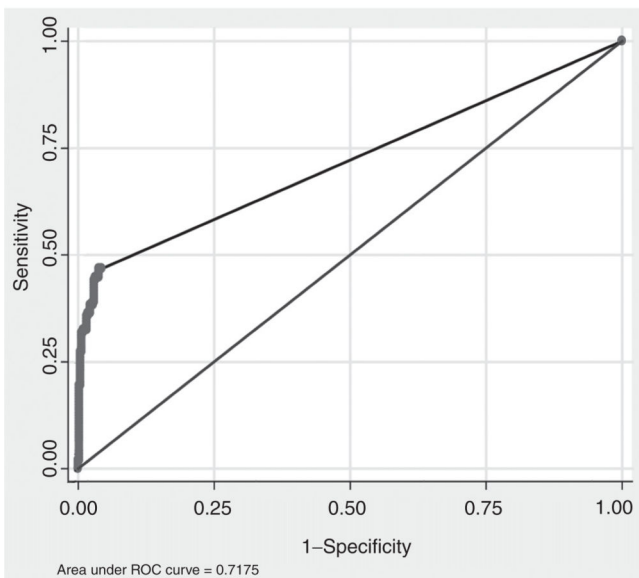
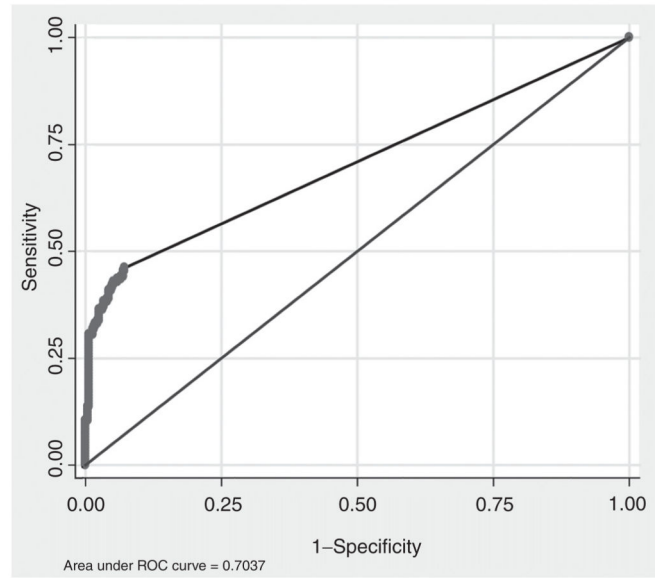
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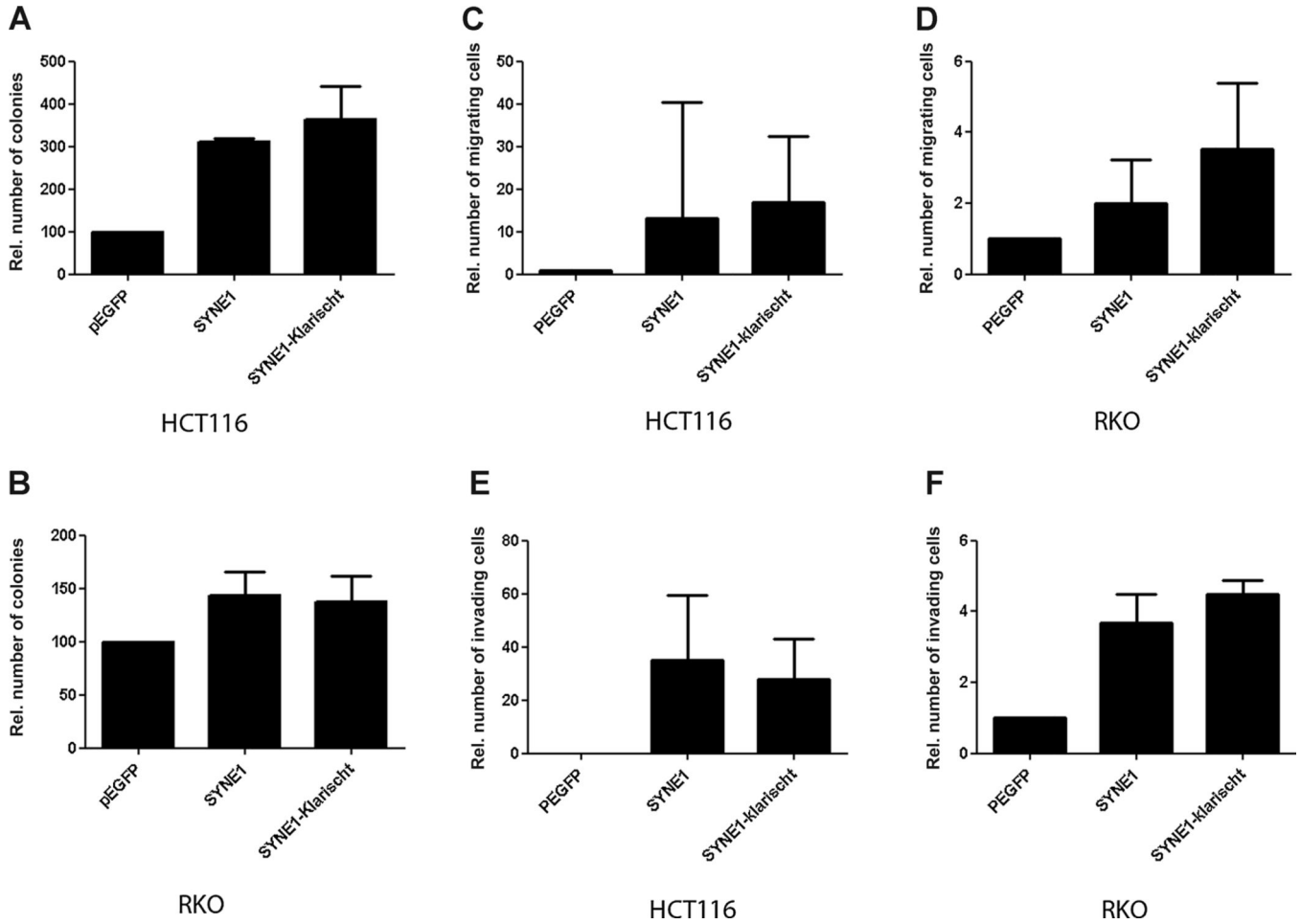
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A NDRG4**B** GATA5**C** SYNE1**D** FOXE1**Figure 1.**

ROC curves. Sensitivity and specificity at various cutoff values for the training set to obtain a positive test for the single markers (A) NDRG4, (B) GATA5, (C) *SYNE1*, and (D) *FOXE1* are shown in the ROC curve.

**Figure 2.**

Functional assays *SYNE1*. A and B, colony formation assay of cells transfected with *SYNE1* expression vector or empty vector grown for 2 weeks in medium containing antibiotics. Mean colony numbers relative to control transfectants are plotted ($n = 3$). Error bars, 95% CI. C and D, invasion assay through Matrigel-coated Transwells. Results represent mean number of *SYNE1*-transfected cells that passed through the Matrigel-coated membranes of the Transwell relative to control cells transfected with empty vector ($n = 3$). E and F, migration assay. Plotted are the mean numbers of *SYNE1*-transfected cells that migrated through Transwell membranes not coated with Matrigel relative to control cells transfected with empty vector ($n = 3$). Error bars, 95% CI.

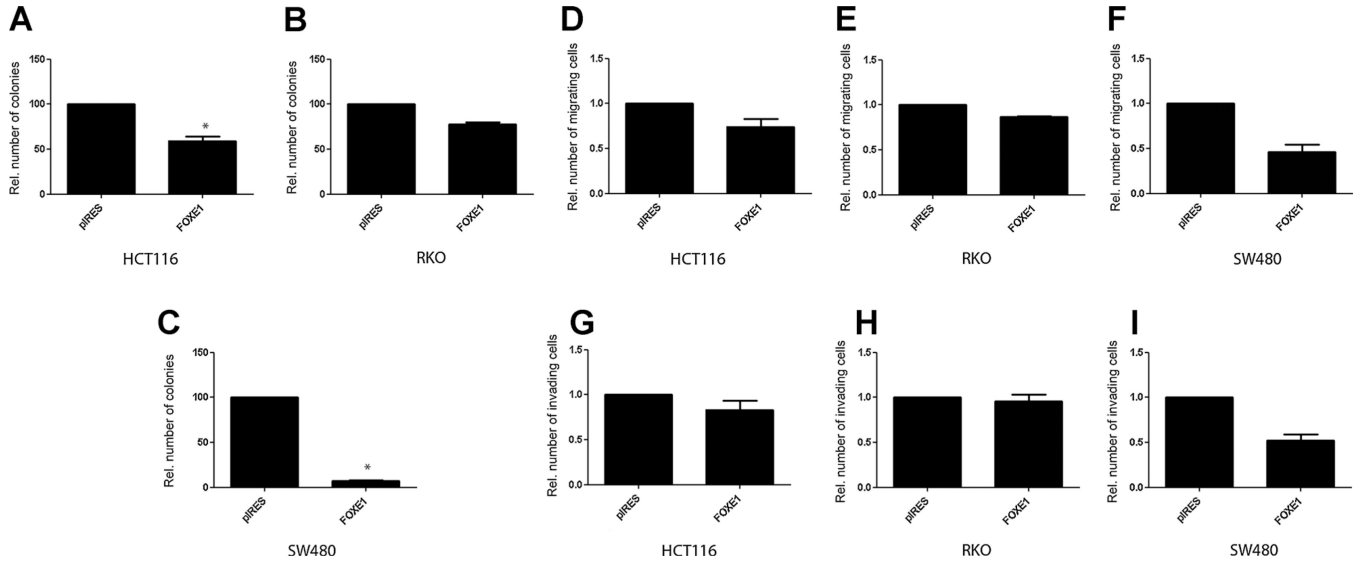


Figure 3. Functional assays FOXE1. A to C, colony formation assay of cells transfected with *FOXE1* expression vector or empty vector grown for 2 weeks in medium containing antibiotics. Mean colony numbers relative to control transfectants are plotted ($n = 3$). Error bars, 95% CI. D to F, invasion assay through Matrigel-coated Transwells. Results represent mean number of *FOXI*-transfected cells that passed through the Matrigel-coated membranes of the Transwell relative to control cells transfected with empty vector ($n = 3$). G to I, migration assay. Plotted are the mean numbers of *FOXI*-transfected cells that migrated through Transwell membranes not coated with Matrigel relative to control cells transfected with empty vector ($n = 3$). Error bars, 95% CI. Melotte et al.

Table 1

Training set plasma performance of single markers [NDRG4 and GATA5 (A); FOXE1 and SYNE1 (B)]

A				
Patient group	NDRG4		GATA5	
	Positive	Sensitivity	Positive	Sensitivity
Stage I	7/43	16%	6/43	14%
Stage II	5/44	11%	4/44	9%
Stage III	16/46	35%	8/46	17%
Stage IV	13/21	62%	10/21	48%
Case total	41/154	27%	28/154	18%
		Specificity		Specificity
Control	22/442	95%	5/444	99%

B				
Patient group	FOXE1		SYNE1	
	Positive	Sensitivity	Positive	Sensitivity
Stage I	15/43	35%	12/43	28%
Stage II	19/44	43%	23/44	52%
Stage III	23/46	50%	21/46	46%
Stage IV	14/21	67%	16/46	76%
Case total	71/154	46%	72/154	47%
		Specificity		Specificity
Control	32/444	93%	19/444	96%

Table 2

Training set results combined FOXE1 and SYNE1

Patient group	SYNE1 and FOXE1	
	Positive	Sensitivity
Stage I	18/43	42%
Stage II	25/44	57%
Stage III	27/46	59%
Stage IV	17/21	81%
Case total	87/154	56%
		Specificity
Control	46/444	90%

Table 3

Test set results combined FOXE1 and SYNE1

Patient group	SYNE1 and FOXE1	
	Positive	Sensitivity
Stage I	10/27	37%
Stage II	13/15	87%
Stage III	11/20	55%
Stage IV	4/4	100%
Case total	38/66	58%
		Specificity
Control	21/240	91%