Methylation of NEUROG1 in Serum Is a Sensitive Marker for the Detection of Early Colorectal Cancer

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OBJECTIVES: **Colorectal cancer is the third most common cancer and a major cause of cancer-related deaths. Early detection of colonic lesions can reduce the incidence and mortality of colorectal cancer. Colonoscopy is the screening test for colorectal cancer with the highest efficacy, but its acceptance in the general public is rather low. To identify suitable tumor-derived markers that could detect colorectal cancer in blood samples, we analyzed the methylation status of a panel of genes in sera of affected patients.**

METHODS: **Using methylation-specific quantitative PCR, we analyzed the methylation of ten marker genes in sera of healthy individuals and patients with colorectal cancer.**

RESULTS: **Only HLTF, HPP1/TPEF, and NEUROG1 DNA methylation was detectable in at least 50% of patients with colorectal cancers. Whereas HLTF and HPP1/TPEF preferentially detected advanced and metastasized colorectal cancers, NEUROG1 methylation was detectable in UICC stages I–IV at a similar rate. Compared with other methylation markers, such as ALX4, SEPT9, and vimentin, NEUROG1 shows a higher sensitivity for colorectal cancer at UICC stages I and II. At a specificity of 91%, NEUROG1 reached a sensitivity of 61% (confidence interval, 50.4–70.6%) for the detection of colorectal cancers. Furthermore, detection of NEUROG1 methylation was independent of age and gender.**

CONCLUSIONS: **Methylation of the *NEUROG1* gene is frequently found in sera of patients with colorectal cancers independent of tumor stage. The quantitative detection of NEUROG1 DNA methylation in serum is a suitable approach for the non-invasive screening for asymptomatic colorectal cancer.**

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/ajg

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INTRODUCTION

Colorectal cancer is the third most common cancer and accounts for ~10 percent of cancer-related deaths overall (1). In addition, one in three people who develop colorectal cancer will ultimately die because of this disease. Detection and removal of early-stage colorectal cancers have been shown to reduce the risk of this tumor (2,3). Over the last decade, colonoscopy has become the preferred screening test to detect colorectal cancer and is endorsed by several national guidelines because it is capable of detecting polyps and cancers with high accuracy (4–6). Apart from this, it is also therapeutic by virtue of its ability to remove polyps and early cancers. Despite its high sensitivity and safety, acceptance of screening colonoscopy remains rather low within the general public, with the need for cathartic bowel preparation being one of the main reasons (7,8). Therefore, sensitive stool- or blood-based tests could be attractive for people who object screening colonoscopy. Stool-based tests such as guaiac-based fecal occult blood tests can reduce colorectal cancer mortality when applied annually or biennially but have limited sensitivity for colorectal cancers and particularly adenomas (9). Furthermore, guaiac-based fecal occult blood test reacts with non-human blood in food, and dietary restrictions are required before sample collection. Immunochemical fecal occult blood tests to specifically detect human occult blood in feces have been developed to overcome the limitations of guaiac-based fecal occult blood tests, but they have not shown better overall test performance compared with guaiac-based fecal occult blood test (4). Stool tests based on the detection of DNA mutations in stool have been proposed and tested in prospective trials (10). However, because of unsatisfying performance, these tests cannot be recommended currently.

Epigenetic DNA modification, such as aberrant hypermethylation, is a common feature of human cancers and is already found in

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Table 1. Characteristics of sample sets

	Pilot set (*n*=47)		Tumor stage set (*n*=95)		Marker comparison set (*n*=61)		Test set (*n*=142)	
	n (male)	Age (min–max)	*n* (male)	Age (min–max)	*N* (male)	Age (min–max)	*n* (male)	Age (min–max)
Healthy	32 (13)	59 (23–80)			16 (9)	53 (23–81)	45 (21)	63[a] (44–81)
UICC I			11 (4)	61 (45–70)	11 (3)	64 (55–77)	27 (13)	64 (44–83)
UICC II			29 (12)	68 (54–84)	9 (5)	68 (47–77)	70 (27)	68 (37–91)
UICC III			37 (16)	66 (41–85)	7 (3)	67 (48–89)		
UICC IV	15 (5)	64 (31–80)	18 (10)	66 (50–83)	18 (6)	63 (47–84)		

Patient serum samples were organized in four different sets: (1) pilot set, (2) tumor stage set, (3) marker comparison set, and (4) test set. The characteristics for each set including sample number (*n*), gender distribution (number of male patients), mean age, and age range (min–max) are shown for healthy persons and patients with colorectal cancers (UICC I–IV).

[a]Data only available for 31 patients.

early stages of carcinogenesis (11). Targets of methylation are CpG islands, regions of DNA within a gene that are characterized by a G + C content ≥50%, and a ratio of observed CpG/expected CpG of 0.5 or greater (12). Regulation of gene expression by aberrant DNA methylation has been widely studied in various cancers including colorectal cancer (13,14).

DNA methylation has not only been studied in primary tumors but also in remote media, including blood and stool (15,16). Interestingly, single stool-based methylation markers have proved a higher sensitivity than genetic tests analyzing panels of gene mutations (17,18). However, blood-based colorectal cancer screening is considered to be the preferred test because storage and processing of blood samples is easier compared with stool samples and patients seem to have a higher acceptance for blood-based tests, which would supposedly lead to higher compliance with this screening test (19). Several marker genes, for example, SEPT9 (20) and ALX4 (21), have been previously described for the detection of asymptomatic colorectal cancers in blood; however, these markers have either a low specificity or large sample volumes are needed for the analysis. Therefore, we tried to identify a sensitive and specific marker for early-stage colorectal cancers using small volumes of serum. Here, we describe the identification of *NEUROG1* (also known as NeuroD3/neurogenin1/NGN1) gene methylation in serum of patients with colorectal cancers. Compared with the methylation of HLTF and HPP1/TPEF (22,23), NEUROG1 methylation is frequently found in serum through all tumor stages but is rarely found in healthy individuals. A side-by-side comparison of NEUROG1 DNA methylation with recently described methylation markers, such as ALX4, SPET9, and vimentin, revealed that NEUROG1 has a higher sensitivity for colorectal cancers at UICC stages I and II than these markers. Our data propose NEUROG1 methylation as a sensitive and specific marker for colorectal cancer detection in serum.

METHODS

Patients and serum samples

Patient serum samples were organized in four different sets: (1) The pilot set comprised healthy individuals and patients with metastasized colorectal cancers (UICC IV). This set was used for the initial screening of 10 potential biomarkers. (2) The tumor stage set contained samples from patients with colorectal cancers (UICC stages I–IV). Only the markers HLTF, HPP1, and NEUROG1 that were positive in at least 50% of the samples in the pilot set were further analyzed in this set. (3) Using sera from healthy donors and patients with colorectal carcinomas, we compared the specificity and sensitivity of NEUROG1 DNA methylation with ALX4, SEPT9, and vimentin methylation in the marker comparison set. (4) The test set comprising serum samples from healthy individuals and colorectal cancers (UICC stages I and II) was used for the analysis of NEUROG1 DNA methylation. **Table 1** contains the characteristics of each set, including sample number, gender distribution, mean age, and age range. The markers analyzed in each group are displayed in **Figure 1**. Healthy individuals were asymptomatic persons that had a colonoscopy and showed no signs of colonic adenomas or carcinomas. Patients with colorectal carcinomas were also examined by colonoscopy before surgery was performed. In each case, serum was separated from blood before endoscopy and classified according to the tumor status. The blood was centrifuged at 3.000×g for 10 min at room temperature, and aliquots were stored at −80 °C. To determine the correlation between the NEUROG1 DNA methylation status in sera and primary colorectal carcinomas, matching samples from 35 patients were used. The ethical committee of the Medical Faculty of the University of Munich approved the study.

DNA isolation and bisulfite conversion of serum samples

Genomic DNA from 1 ml of each serum sample was isolated using the QIAamp DNA Blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sodium bisulfite conversion of genomic DNA was done as described previously (15). Briefly, isolated serum DNA was spiked with salmon sperm DNA and denatured using alkaline conditions. DNA was treated with sodium bisulfite for 16 h at 55 °C and purified using the Wizard DNA Clean-up system (Promega, Madison, WI, USA). After ethanol precipitation, bisulfite-treated DNA was resuspended in 30 µl Tris-HCl (1 mM (pH 8.0)).

DNA isolation and bisulfite treatment of tissue samples

Paraffin-embedded tissue samples of 35 patients of whom serum samples were also available were collected. Serial sections were

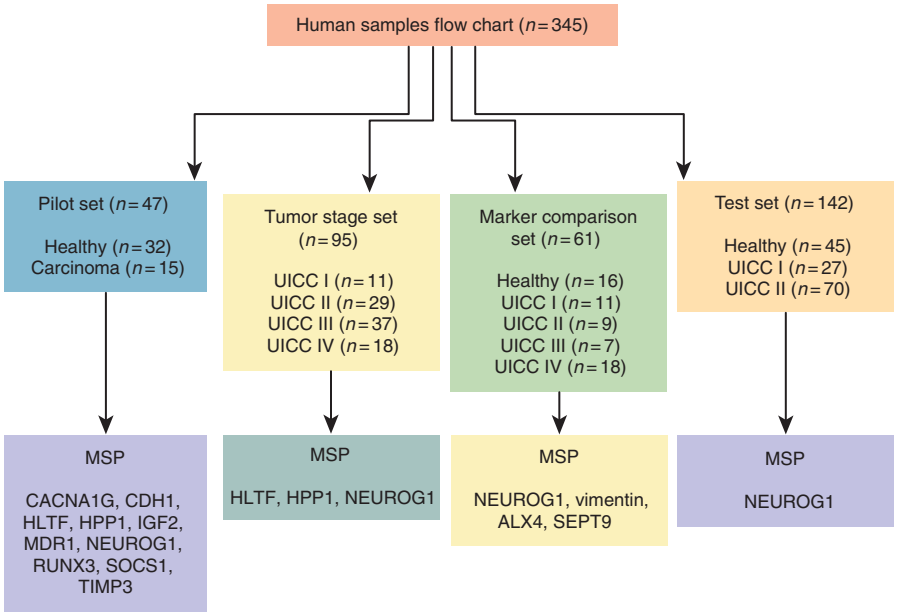

Figure 1. Samples used for the identification of a suitable serum marker. In total, 345 samples were analyzed to identify a suitable serum marker for the detection of colorectal cancer. The pilot set comprised healthy individuals and patients with metastasized colorectal carcinomas (UICC IV). The 10 genes listed were screened using quantitative PCR. The tumor stage set contained colorectal carcinomas (UICC stages I–IV) and three genes—*NEUROG1, HLTF,* and *HPP1*—were analyzed in this set. The marker comparison set was used to compare the detection of methylated DNA for the markers NEUROG1, ALX4, SEPT9, and vimentin in sera of healthy individuals and patients with colorectal carcinomas. To verify the results obtained in the pilot and tumor stage sets, the marker NEUROG1 was analyzed in a test set containing samples from healthy individuals and patients with colorectal carcinomas (UICC I and II). MSP, methylation-specific PCR.

performed of these tissue samples. Hematoxylin and eosin staining was done for one slide, which was then inspected by a pathologist who marked the tumor region. Tumor tissue was scraped from a deparaffinized, adjacent slide using the hematoxylin and eosin-stained slide as blueprint. DNA was purified using the QIAamp DNA FFPE Tissue Kit (Qiagen). Proteinase K incubation at 56 °C was done overnight. The following incubation step at 90 °C was omitted. Subsequent steps were performed following the manufacturer's protocol.

Sodium bisulfite conversion of DNA was performed using the EZ DNA Methylation-Gold Kit (Zymo Research, Freiburg, Germany) according to the manufacturer's protocol. In all, 2 µl of the eluted DNA was used for each subsequent MethyLight PCR reaction.

Analysis of DNA methylation

Bisulfite-treated DNA was analyzed by a fluorescence-based, real-time PCR assay, described previously as MethyLight (24,25). Briefly, two sets of primers and probes, designed specifically to bind to bisulfite-converted DNA, were used: one set of primers for every methylated target to be analyzed (**Table 2**) and a pair of primers for Alu repeat sequences (26) to control for DNA amplification and normalize for input DNA. Specificity of the reactions for methylated DNA was confirmed by separately amplifying completely methylated and unmethylated human control DNA (Chemicon, Temecula, CA) with each set of primers and probes. PCRs were carried out in 20 µl volumes containing 1× PCR buffer (Qiagen), 1.25 mM $MgCl_2$, 250 µM dNTP mixture, 0.5 µM of each primer, 0.3 µM of each probe, 1x Q-Solution (Qiagen), 2 µl bisulfite-treated DNA, and 0.05 U/µl Taq DNA polymerase (HotStar Taq, Qiagen). PCRs were carried out in a Mastercycler realplex 4S (Eppendorf, Hamburg, Germany) using the following conditions: 95 °C for 900 s, followed by 40 cycles of 94 °C for 30 s, 60 °C for 120 s, and 84 °C for 20 s. The number of molecules for each gene of interest and Alu repeat sequences were calculated using a standard curve. The number of molecules for each gene of interest was then normalized to the number of molecules for the Alu repeat sequences.

Statistical analysis

Pearson's χ^2-test and Fisher's exact test were used to analyze the gender distribution. Mann–Whitney test and one-way analysis of variance were used to analyze age distribution in the three sets of samples. Pearson's χ^2-test and Fisher's exact test were used to explore associations between clinicopathological features. All these statistical analyses were performed using the software Prism 4 (GraphPad Software, San Diego, CA).

To generate the receiver operator characteristic (ROC) curve for the marker NEUROG1, the sensitivity for each possible threshold value in the range from 0 and 1 was calculated and graphed against the corresponding value of 1 − specificity. If the area under the ROC curve corresponds to the bisecting line (area under the curve equals 0.5), the test (and the tested marker) is useless. However, the test gets better, the further the ROC curve moves to the upper left corner (ideal value for the area under the curve equals 1). Whereas the area under the curve defines the quality of the ROC analysis, the Youden index defines the optimal threshold value.

Table 2. Primers and probes used for analysis of DNA methylation in serum.

Gene ID	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Probe oligo sequence (5′ FAM/3′ BHQ; 5′–3′)
ALX4	CGTCGCAACGCGTACG	CGCGGTTTCGATTTTAATGC	ACTCCGACTTAACCCGACGATCG
CACNAG1	TTTTTTCGTTTCGCGTTTAGGT	CTCGAAACGACTTCGCCG	AAATAACGCCGAATCCGACAACCGA
CDH1	ATTGTAAAGTATTTGTGAGTTTGCG	AATACCTACAACAACAACAACAACG	CGTTGTTGATTGGTTGTGGTCGGTAGGT
HLTF	CGGCGTTCGGAATTTGTT	AAACGCCTCGACTCCCCTAA	AGGAGGCGTATCGAGGCGGTTCG
HPP1	GTTATCGTCGTCGTTTTTGTTGTC	GACTTCCGAAAAACACAAAATCG	CCGAACAACGGACTACTAAACATCCCGCG
IGF2	GAGCGGTTTCGGTGTCGTTA	CCAACTCGATTTAAACCGACG	CCCTCTACCGTCGCGAACCCGA
MDR1	CGGTTTAGGACGCGAAAAGAT	TCCTTCTTAACGCGTCTAATACCA	AGGTTGAGGTGGGAGCGATATGGTGTG
NeuroG1	CGTGTAGCGTTCGGGTATTTGTA	CGATAATTACGAACACACTCCGAAT	CGATAACGACCTCCCGCGAACATAAA
RUNX3	CGTTCGATGGTGGACGTGT	GACGAACAACGTCTTATTACAACGC	CGCACGAACTCGCCTACGTAATCCG
SEPT9	AAATAATCCCATCCAACTA	TTAACCGCGAAATCCGAC	GATT**X**GTTGTTTATTAGTTATTATGT
SOCS1	GCGTCGAGTTCGTGGGTATTT	CCGAAACCATCTTCACGCTAA	ACAATTCCGCTAACGACTATCGCGCA
TIMP3	TTCGCGTGTTTACGGCG	GCCCCCTCAAACCAATAACAA	TTTGGAGGGTTGATGAGGTAATGCGGT
Vimentin	ATAGTTTGGGTAGCGCGTTG	ACGTCGACCAACGAAAAATC	TTTTACGTTTCGTTTTCGGG

At this index point, the sum consisting of sensitivity and the specificity reaches a maximum. This statistical analysis was performed using SAS for Windows software version 9.2 (SAS Institute, Cary, NC).

RESULTS

Identification of genes frequently methylated in the serum of patients with colorectal cancers

To identify sensitive and specific serum markers for early-stage colorectal cancer, we performed a literature search for genes that have been previously reported to be frequently hypermethylated in primary colorectal cancers and identified 10 candidate genes: *CACNA1G, CDH1, HLTF, HPP1/TPEF, IGF2, MDR1, NEUROG1, RUNX3, SOCS1,* and *TIMP3* (27–29). As we expected the highest frequencies of methylation for marker genes in advanced disease stages and only markers with high frequencies of methylation in advanced stages are likely to be methylated in early-stage cancer, we decided to use International Union Against Cancer (UICC) stage IV colorectal cancers for the initial search for markers. Consequentially, we analyzed the DNA methylation of the 10 genes in sera of 32 healthy individuals and 15 patients with advanced colorectal cancers. Based on the normalized number of DNA molecules for each gene of interest (**Supplementary Figure 1** online), we defined four threshold values (>0, >0.005, >0.05, and >0.1) and calculated the sensitivity and specificity for each gene using these thresholds (**Table 3**). We arbitrarily chose a detection rate of at least 50% and a false positive rate of < 10% as a starting point for the identification of suitable marker genes. Only three of the genes tested fit these criteria: methylation of HLTF was found in 3.1% of healthy individuals and 60% of cancer sera (threshold value >0.005). HPP1/TPEF methylation was detected in 6.3% of healthy individuals and 66.7% of cancer patients (threshold value >0). NEUROG1 was positive in 6.7% of healthy individuals and 93.3% of cancer patients (threshold value >0.05).

NEUROG1 methylation in serum is frequently found in all stages of colorectal cancers

To test whether the three identified markers, NEUROG1, HLTF, and HPP1/TPEF, were also capable of identifying cancers at earlier stages and not only advanced cases, sera of 95 patients with colorectal cancers of different stages (UICC stages I, II, III, and IV) were analyzed. HLTF methylation was found altogether in 10 (10.5%) cases, *HPP1/TPEF* gene methylation was detected in 19 (20%) cases. Furthermore, the number of HLTF- and HPP1/TPEF-positive cases increased starting from UICC stage I–IV (**Figure 2a** and **b**). In contrast, NEUROG1 methylation was found overall in 63 of the 95 cases (or 66.3%), and no significant difference in the number of NEUROG1-positive cases was observed between UICC tumor stages I and IV (analysis of variance, $P = 0.769$; **Figure 2c**). Therefore, only *NEUROG1* gene serum methylation would qualify as a potential diagnostic marker for early colorectal cancers.

To find out whether the NEUROG1 DNA methylation status of the serum matched the NEUROG1 methylation status of the primary colorectal carcinoma, 35 primary colorectal cancers and matching serum from the same patients were analyzed. We found 18 NEUROG1 methylation-positive sera and 25 NEUROG1 methylation-positive tumors. Comparing the NEUROG1 methylation status on an individual basis revealed that sera and tumor were either both positive (13 out of 35 cases) or negative (5 out of 35 cases). In 12 cases, methylated NEUROG1 could only be detected in the tumor but not in the serum, whereas in 5 cases, the NEUROG1 methylation was found only in the serum.

To test the sensitivity of NEUROG1 in comparison with other previously proposed DNA methylation markers, such as ALX4, SEPT9, and vimentin, we determined the detection rates of NEUROG1, ALX4, SEPT9, and vimentin in sera of 16 healthy individuals and 45 patients with colorectal cancer. In this marker comparison set, the marker NEUROG1 had an overall specificity of 81.3% and sensitivity of 55.5% compared with

Table 3. Identification of suitable diagnostic serum markers (pilot set)

Threshold status	>0		>0.005		>0.05		>0.1	
	Normal	Carcinoma	Normal	Carcinoma	Normal	Carcinoma	Normal	Carcinoma
No. of samples	*n*=32	*n*=15	*n*=32	*n*=15	*n*=32	*n*=15	*n*=32	*n*=15
CACNA1G*	20.0 (4.3–48.1)	20.0 (4.3–48.1)	13.3 (1.7–40.5)	20.0 (4.3–48.1)	0.0 (0.0–21.8)	6.7 (2.0–31.9)	0.0 (0.0–21.8)	0.0 (0.0–21.8)
CDH1	3.1 (0.1–14.2)	0.0 (0.0–21.8)	0.0 (0.0–9.5)	0.0 (0.0–21.8)	0.0 (0.0–9.5)	0.0 (0.0–21.8)	0.0 (0.0–9.5)	0.0 (0.0–21.8)
HLTF	9.4 (2.0–25.0)	66.7 (38.4–88.2)	3.1 (0.1–14.2)	60.0 (32.3–83.7)	0.0 (0.0–9.5)	26.7 (7.8–55.1)	0.0 (0.0–9.5)	26.7 (7.8–55.1)
HPP1/TPEF	6.3 (0.7–18.2)	66.7 (38.4–88.2)	0.0 (0.0–9.5)	46.7 (21.3–73.4)	0.0 (0.0–9.5)	20.0 (4.3–48.1)	0.0 (0.0–9.5)	13.3 (1.7–40.5)
IGF2*	60.0 (32.3–83.7)	53.3 (26.6–78.7)	40.0 (16.3–67.7)	40.0 (16.3–67.7)	0.0 (0.0–21.8)	6.7 (2.0–31.9)	0.0 (0.0–21.8)	6.7 (2.0–31.9)
MDR1	3.1 (0.1–14.2)	6.7 (2.0–31.9)	0.0 (0.0–9.5)	0.0 (0.0–21.8)	0.0 (0.0–9.5)	0.0 (0.0–21.8)	0.0 (0.0–9.5)	0.0 (0.0–21.8)
NEUROG1*	80.0 (51.9–95.7)	100.0 (78.2–100.0)	26.7 (7.8–55.1)	100.0 (78.1–100.0)	6.7 (2.0–31.9)	93.3 (68.1–99.8)	6.7 (2.0–31.9)	86.7 (59.5–98.3)
RUNX3*	13.3 (1.7–40.5)	40.0 (16.3–67.7)	13.3 (1.7–40.5)	13.3 (1.7–40.5)	0.0 (0.0–21.8)	0.0 (0.0–21.8)	0.0 (0.0–21.8)	0.0 (0.0–21.8)
SOCS1*	13.3 (1.7–40.5)	33.3 (11.8–61.6)	6.7 (2.0–31.9)	20.0 (4.3–48.1)	0.0 (0.0–21.8)	0.0 (0.0–21.8)	0.0 (0.0–21.8)	0.0 (0.0–21.8)
TIMP3	6.3 (0.7–18.2)	20.0 (4.3–48.1)	3.1 (0.1–14.2)	13.3 (1.7–40.5)	0.0 (0.0–9.5)	0.0 (0.0–21.8)	0.0 (0.0–9.5)	0.0 (0.0–21.8)

The sensitivities for the indicated marker genes were calculated in colorectal carcinomas and healthy controls for four different threshold values. Case numbers (*n*) are shown for each group. Marker genes marked with an asterisk were analyzed in 15 healthy controls only. 95% confidence intervals are given in brackets.

ALX4 (specificity: 66.3%; sensitivity: 46.6%), SEPT9 (specificity: 81,3%; sensitivity: 46.6%) and vimentin (specificity: 60%; sensitivity: 31.1%) (**Figure 3**). Analyzing patients with colorectal cancer in the UICC tumor stages I and II, NEUROG1 was detected in 11 out of 20 (55%) samples, confirming our results from the tumor stage set. This detection rate of NEUROG1 for UICC I and II cancers was higher than for ALX4 (30%), SEPT9 (20%), and vimentin (20%). Analyzing tumors in UICC stages III and IV revealed that ALX4 and SEPT9 detected more colorectal cancers with 15 out of 25 (60%) for ALX4 and 16 out of 25 (64%) for SEPT9 compared with 14 out of 25 (56%) for NEUROG1. Taken together, these data suggest that detection of methylated NEUROG1 DNA in serum is a suitable marker for the detection of colorectal cancer at early stages.

NEUROG1 DNA methylation can be detected in sera of patients with early-stage colorectal cancers

Based on the threshold value of >0.05 defined for NEUROG1 in the pilot set (**Table 3**), we determined the number of NEUROG1-positive samples in a test set with 45 healthy controls and 97 patients with early-stage colorectal carcinomas (UICC I and II) (**Table 4**, fifth column). Using this threshold, we found a sensitivity of 51.9% for UICC I and 67.1% for UICC II at a specificity of 80%. To calculate the optimal threshold value for the marker NEUROG1, a ROC curve was generated using the 45 samples from healthy controls and 97 samples from patients with colorectal carcinomas (**Figure 4**). The resulting area under the curve was 0.730 (95% confidence interval, 0.649–0.811). The calculated threshold value (Youden index) of 0.089 was used to determine the number of NEUROG1-positive samples (**Table 4**, eighth column) and to compute the sensitivity of the marker NEUROG1 for colorectal carcinomas as well as the specificity: at a specificity of 91.1%, *NEUROG1* gene methylation was found in 51.9% of patients with stage I cancers (14 of 27 cases) and 64.3% of stage II cancers (45 of 70 cases) (**Figure 5**). Increasing the threshold value for NEUROG1 from >0.05 to >0.089 improved the specificity in the test set without a relevant change in sensitivity for carcinomas. Furthermore, statistical analysis of the test set clearly demonstrated that the detection of the methylation marker NEUROG1 is associated with colorectal carcinomas (analysis of variance, $P < 0.0001$; **Table 4**). All other criteria tested including gender, age, localization, and stage of the tumors did not reveal a significant correlation with *NEUROG1* gene methylation in serum.

DISCUSSION

Despite recent advances, colorectal cancer is one of the most frequent occurring cancers with a high mortality rate. Early detection and removal of precancerous lesions can reduce the incidence and mortality of colorectal cancer, and several approaches to detect this tumor at early stages have been established. Colonoscopy is currently the most efficient technique (30,31). However, the acceptance for this screening test in the general public is low. For this reason, we tried to identify markers that are capable of detecting asymptomatic colorectal cancers in small volumes of serum. Here, we show that NEUROG1 DNA methylation represents a suitable diagnostic serum marker that discriminates between healthy individuals and individuals with colorectal cancer at early

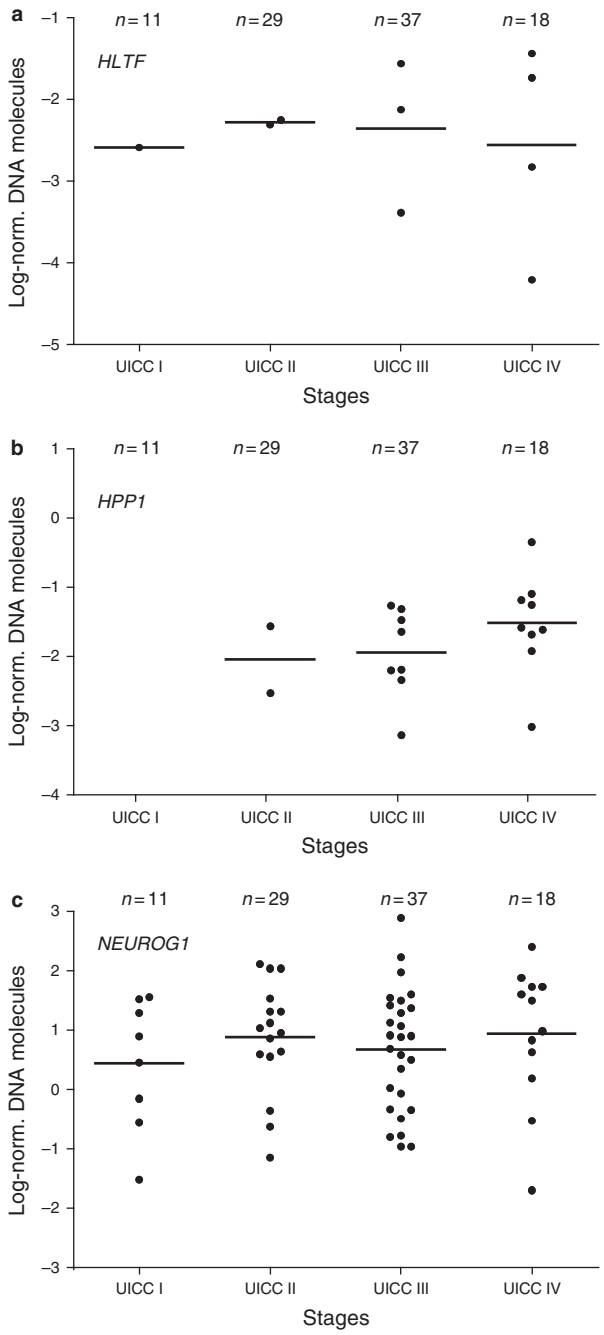

Figure 2. Analysis of DNA methylation for the marker genes *HLTF, HPP1,* and *NEUROG1* at different tumor stages. The graph depicts the normalized number of methylated DNA molecules for the indicated marker genes, *HLTF* (**a**), and *HPP1* (**b**), and *NEUROG1* (**c**) at the UICC stages I to IV. Each dot represents one methylation-positive tumor sample (total number of samples UICC I–IV: 95). A horizontal line symbolizes the average number of normalized DNA molecules for each tumor stage. Case numbers (*n*) are displayed for each tumor stage. norm., normalized.

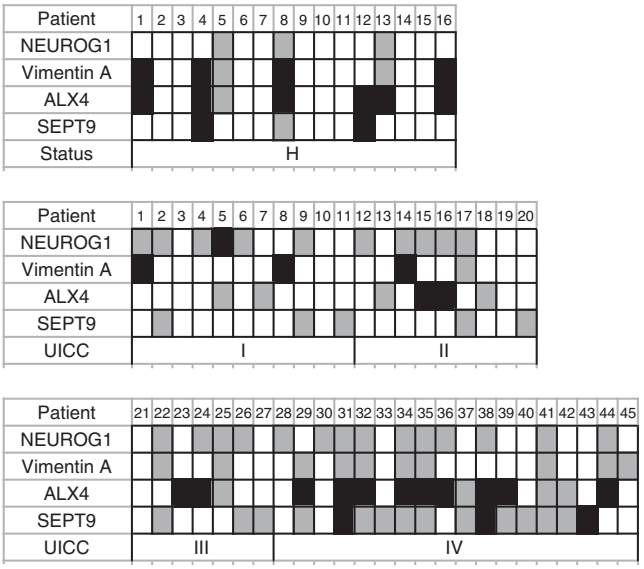

Figure 3. Comparison of NEUROG1, ALX4, SEPT9, and vimentin serum DNA methylation. Serum DNA methylation for each marker was measured by quantitative PCR in sera from healthy individuals (H) and patients with colorectal cancer (UICC I–IV). The number of methylated DNA molecules for each marker was normalized to the reference gene *ALU*. Normalized DNA numbers for each marker were translated into a color code with white (=0), light grey (0< ×<1), medium grey (1–100), and black (>100). The samples were sorted according to tumor stage.

stages. Healthy persons have very low levels of methylated NEUROG1 DNA in their sera; however, there is a significant increase in methylated NEUROG1 DNA in serum of patients with colorectal cancer. This change in the NEUROG1 methylation status can be used to identify patients with early-stage colorectal cancers that would otherwise remain undetected. Our observation that HLTF and HPP1/TPEF are predominantly found in advanced cancers is in accordance with our previous findings that the methylation status of these genes can be used as prognostic markers (22,23).

Abnormally high levels of free circulating DNA in plasma/serum of cancer patients have been described for the first time over 30 years ago (32). Even though the underlying mechanism for the generation of the circulating DNA has not been resolved yet, Vasioukhin *et al.* (33) and Sorenson *et al.* (34) demonstrated that DNA mutations (for N-Ras and K-Ras, respectively) found in tumor cells can also be detected in the free circulating DNA in serum. To address the question whether the methylated NEUROG1 DNA found in the sera was released from colorectal carcinomas, we determined the methylation status of NEUROG1 in sera and matched tumor tissues of 35 patients with colorectal carcinomas. In 18 of these cases, we found a positive correlation between the NEUROG1 status in the serum and tumor tissue, whereas in 12 cases, NEUROG1 DNA methylation was only detectable in the tumor tissue, but not in the serum. The reason for this discrepancy could be low DNA turnover or poor vascularization of the tumor, preventing the release of (methylated) tumor DNA into the blood stream. In a small number of samples, we detected NEUROG1 DNA methylation in the serum without a corresponding NEUROG1 status in the carcinoma. Although we can only speculate about the reasons for these differences, failure to detect NEUROG1 methylation in some primary tumors is most likely caused by a heterogeneous distribution of methylation within the tumor itself (35). Therefore, analysis of a fraction of the tumor only might result in false negative results.

Table 4. Hypermethylated NEUROG1 DNA was detectable in early-stage colorectal carcinomas

Status	Characteristic		No. of sample	Threshold > 0.05			Threshold > 0.089		
				No. of NEUROG1-positive samples		*P*	No. of NEUROG1-positive samples		*P*
Healthy	Gender	Male	21	6	28.6%	0.267	3	14.3%	0.326
		Female	24	3	12.5%		1	4.2%	
	Age[a]	×<60	12	4	33.3%	0.704	3	25.0%	0.272
		×≥60	19	5	26.3%		1	5.3%	
Carcinoma	Gender	Male	40	26	65.0%	0.832	26	65.0%	0.531
		Female	57	35	61.4%		33	57.9%	
	Age	×<60	31	21	67.7%	0.653	21	67.7%	0.380
		×≥60	66	40	60.6%		38	57.6%	
	Localization	Proximal	27	14	51.9%	0.366	13	48.1%	0.249
		Distal	38	26	68.4%		26	68.4%	
		Rectal	32	21	65.6%		20	62.5%	
	Stage	UICC I	27	14	51.9%	0.170	14	51.9%	0.356
		UICC II	70	47	67.1%		45	64.3%	
	Disease	Healthy	45	9	20.0%	<0.0001	4	8.9%	<0.0001
		Carcinoma	97	61	62.9%		59	60.8%	

To test the correlation of NEUROG1-positive samples with various parameters, Pearson's χ^2-test was applied to samples of the test set. Proximal localizations of the tumor included cecum up to (and including) the transverse colon, whereas tumor localizations distal of the transverse colon were classified as distal.

[a]Data only available for 31 out of 45 samples.

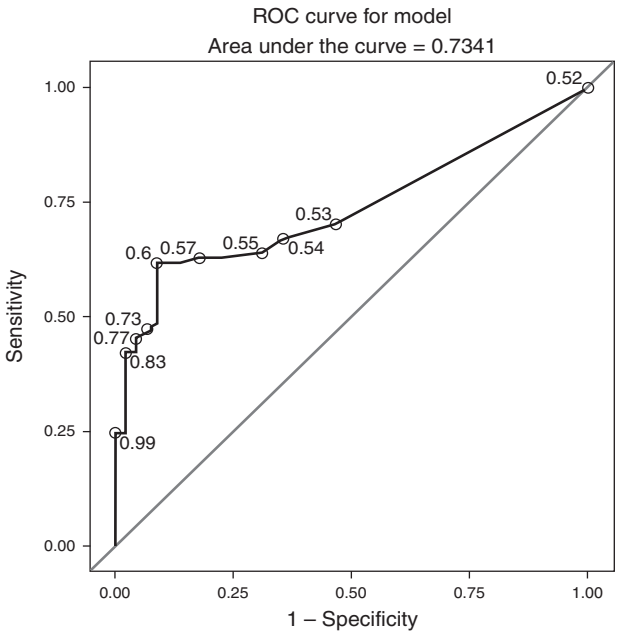

Figure 4. Receiver operator characteristic (ROC) curve for the molecular marker NEUROG1. A ROC curve was generated using the test set with samples from 45 healthy controls and 97 patients with colorectal carcinomas (UICC I and II) to determine the optimal threshold value for the marker NEUROG1.

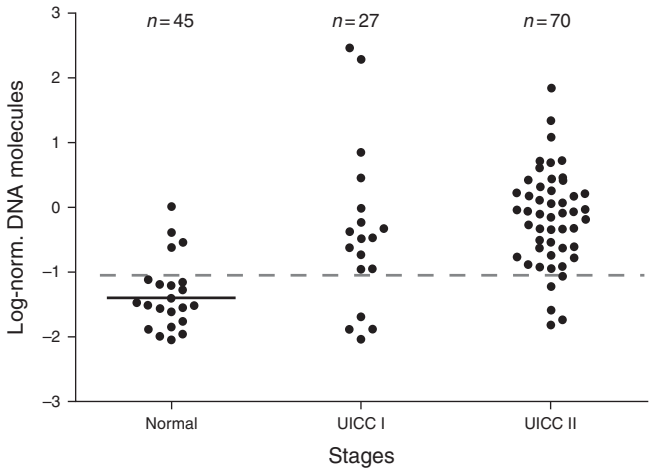

Figure 5. Analysis of serum DNA methylation for the marker gene *NEUROG1*. The graph depicts the normalized number of methylated DNA molecules for the marker gene *NEUROG1* in 45 healthy individuals and 97 colorectal cancers (UICC stages I and II). Each dot represents one methylation-positive sample. Horizontal black lines symbolize the average number of normalized DNA molecules. The gray-dashed line depicts the threshold value of 0.089 as determined by the receiver operator characteristic (ROC) analysis. Case numbers (*n*) are displayed for each tumor stage. norm., normalized.

The analysis of methylated DNA markers in peripheral blood has the advantage over the analysis of fecal DNA markers that blood storage and processing is easier compared with the handling of stool samples. Owing to contamination with bacterial DNA, the analysis of methylation in stool samples requires extensive sample processing and, for this reason, is more laborious than analysis of

the same markers in blood (19). This statement holds also true for the detection of mutated DNA in stool samples, an approach showing an unsatisfying performance at this time (10). Apart from this, there seems to be a higher acceptance for blood-based tests compared with colonoscopy in the general public, possibly resulting in higher compliance rates for routine screening procedures (19).

For the detection of methylated NEUROG1 DNA, we used 1 ml of serum from healthy persons and patients with colorectal cancer. Under these conditions, we found a sensitivity of 51.9% for patients with stage I cancers and 64.3% for patients with stage II cancers at a specificity of 91.1%. With these values, the marker NEUROG1 has similar qualities as the methylation marker gene *SEPT9* that has been reported to have a specificity of 86% and a sensitivity of 69% (36). Another potentially interesting methylated DNA marker, ALX4, has a higher sensitivity (83%) compared with NEUROG1, but its specificity of 70% lags far behind (21). Similar to NEUROG1, methylation of the marker vimentin in plasma samples has been described with a sensitivity of 59% and a specificity of 93% (37).

A side-by-side comparison of NEUROG1, ALX4, SEPT9, and vimentin DNA methylation in sera revealed that the markers ALX4 and SEPT9 have a slightly higher sensitivity than NEUROG1 toward colorectal cancers at UICC stages III and IV. However, NEUROG1 alone performed better when colorectal cancers at UICC stages I and II were analyzed, suggesting that NEUROG1 is a superior marker for the detection of colorectal cancer at early stages.

The number of NEUROG1 methylation-positive samples is higher in the pilot set than in the other sets. We therefore compared the number of methylated NEUROG1 molecules in the positive samples of the pilot set with the other sets. There was no difference between sets, suggesting that DNA extraction and PCR conditions were similar. However, the detection rates for HLTF and HPP1 were also higher in the pilot set than in the tumor stage set. Therefore, it is most likely that random selection of samples have led to inclusion of more "positive" samples by chance than in the other sets. A larger sample size would probably have prevented this. However, as NEUROG1, HLTF, and HPP1 were detected methylated at higher rates in the pilot set compared with the other sets, this has most likely not had an influence on the marker selection. The later sets consistently demonstrate NEUROG1 sensitivities of 50–60% in sera of UICC IV patients.

Compared with FOBTs, which have sensitivities for colorectal carcinomas around 30% and specificities of 90% (38), detection of NEUROG1 DNA methylation in sera shows a higher sensitivity for colorectal carcinomas (55%) while having a similar specificity (91%). In addition, as blood samples can be taken on a routine basis and people in general seem to favor blood tests over stool tests, detection of NEUROG1 DNA methylation could be an interesting alternative to FOBTs. However, before the marker NEUROG1 can be employed for routine clinical application, it has to demonstrate its qualities in a prospective clinical trial.

In conclusion, we have identified NEUROG1 DNA methylation as a sensitive and specific marker for the remote detection of early-stage colorectal cancer in small volumes of serum. NEUROG1 holds the potential to be used as a marker—either alone or in combination with other markers—for the non-invasive and population-wide screening for colorectal neoplasia, especially for persons who refuse screening colonoscopy.

CONFLICT OF INTEREST

Guarantors of the article: Andreas Herbst, PhD and Frank T. Kolligs, MD.

Specific author contributions: Sample collection and experiments: Konstanze Rahmig, Petra Stieber, Alexander Philipp, Andrea Ofner, Andreas Jung, Jens Neumann, and Rolf Lamerz; data analysis and interpretation: Andreas Herbst, Alexander Crispin, and Frank T. Kolligs; study design and preparation of the manuscript: Andreas Herbst and Frank T. Kolligs.

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Potential competing interests: None.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ Colonoscopy is the preferred screening test to detect colorectal cancer.
- ✓ Colonoscopy suffers from low acceptance in the general public.
- ✓ Blood-based screening tests are an interesting alternative.

WHAT IS NEW HERE

- ✓ Methylated NEUROG1 DNA is a sensitive and specific marker for colorectal cancer.
- ✓ Methylated NEUROG1 DNA can be detected in small volumes of serum.
- ✓ Suitable for persons who have objections against colonoscopy.

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