

N-Myc downstream-regulated gene 4 (NDRG4): a candidate tumor suppressor gene and potential biomarker for colorectal cancer

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N-Myc Downstream-Regulated Gene 4 (*NDRG4*): A Candidate Tumor Suppressor Gene and Potential Biomarker for Colorectal Cancer

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- Background** Identification of hypermethylated tumor suppressor genes in body fluids is an appealing strategy for the noninvasive detection of colorectal cancer. Here we examined the role of N-Myc downstream-regulated gene 4 (*NDRG4*) as a novel tumor suppressor and biomarker in colorectal cancer.
- Methods** *NDRG4* promoter methylation was analyzed in human colorectal cancer cell lines, colorectal tissue, and noncancerous colon mucosa by using methylation-specific polymerase chain reaction (PCR) and bisulfite sequencing. *NDRG4* mRNA and protein expression were studied using real-time-PCR and immunohistochemistry, respectively. Tumor suppressor functions of *NDRG4* were examined by colony formation, cell proliferation, and migration and invasion assays in colorectal cancer cell lines that were stably transfected with an *NDRG4* expression construct. Quantitative methylation-specific PCR was used to examine the utility of *NDRG4* promoter methylation as a biomarker in fecal DNA from 75 colorectal cancer patients and 75 control subjects. All *P* values are two-sided.
- Results** The prevalence of *NDRG4* promoter methylation in two independent series of colorectal cancers was 86% (71/83) and 70% (128/184) compared with 4% (2/48) in noncancerous colon mucosa (*P* < .001). *NDRG4* mRNA and protein expression were decreased in colorectal cancer tissue compared with noncancerous colon mucosa. *NDRG4* overexpression in colorectal cancer cell lines suppressed colony formation (*P* = .014), cell proliferation (*P* < .001), and invasion (*P* < .001). *NDRG4* promoter methylation analysis in fecal DNA from a training set of colorectal cancer patients and control subjects yielded a sensitivity of 61% (95% confidence interval [CI] = 43% to 79%) and a specificity of 93% (95% CI = 90% to 97%). An independent test set of colorectal cancer patients and control subjects yielded a sensitivity of 53% (95% CI = 39% to 67%) and a specificity of 100% (95% CI = 86% to 100%).
- Conclusions** *NDRG4* is a candidate tumor suppressor gene in colorectal cancer whose expression is frequently inactivated by promoter methylation. *NDRG4* promoter methylation is a potential biomarker for the noninvasive detection of colorectal cancer in stool samples.

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Hypermethylation of CpG islands in the promoter region of genes is associated with gene silencing, may serve as a mechanism to inactivate tumor suppressor genes in colorectal cancer carcinogenesis, and can be analyzed easily by using methylation-specific polymerase chain reaction (PCR). Identification of methylation markers that are sensitive and specific for colorectal cancer detection may improve the early detection of this disease. Previous microarray experiments (1) to identify genes that are epigenetically regulated

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in tumor endothelium revealed 81 genes whose expression was decreased in tumor endothelial cells compared with quiescent endothelial cells and that were reexpressed after treatment with the DNA methylation inhibitor 5-aza-2'-deoxycytidine (DAC) and the histone deacetylase inhibitor trichostatin A. Silencing of these genes in tumor endothelium was associated with modifications of histones in the promoter regions but not with promoter CpG island methylation (1). It is interesting that 21 (26%) of the 81 genes were reported to be hypermethylated at their promoters and silenced in various tumor types, suggesting that expression of those genes in tumor cells may be regulated by promoter methylation (1).

One of the identified genes was N-Myc downstream-regulated gene 4 (*NDRG4*; also known as *SMAP-8* and *BDMI*). The protein encoded by this gene, NDRG4, is a member of the NDRG protein family, which comprises four members named NDRG1–4 that have 57%–65% amino acid sequence homology (2,3). NDRG1 is the most extensively studied member of the NDRG family. Expression of NDRG1 is decreased in cancer cells (4–9) but increases in cancer cells that are treated with DAC (5,6). It has been demonstrated that NDRG1 overexpression in colorectal cancer cell lines reduces their ability to metastasize in nude mice, and the NDRG1-mediated suppression of metastasis is thought to involve the induction of colorectal cancer cell differentiation and a partial reversal of the metastatic phenotype (6). *NDRG2* has been described as a candidate tumor suppressor gene (10,11) and displays promoter CpG island methylation in meningiomas (10) and in breast, liver, and lung cancer cell lines (12). To our knowledge, the roles of NDRG3 and NDRG4 in cancer have not been addressed.

The *NDRG4* gene is located at chromosome 16q21–q22.3, spans 26 kilobases, and contains 17 exons that include the entire sequence of the three complementary DNA (cDNA) isoforms: *NDRG4-B*, *NDRG4-B^{var}*, and *NDRG4-H* (2). To our knowledge, NDRG4 expression has only been described in the brain and heart using northern blot analysis. The molecular characterization of NDRG4 and the role of this protein in the nervous system have been investigated mainly in the rat (13–16), where it is thought to participate in processes that lead to cellular differentiation and neurite formation (14).

Here we examined the expression of *NDRG4* at the mRNA and protein levels in normal human colon mucosa and human colorectal cancer tissue. In addition, we examined the mechanism underlying the decreased expression of *NDRG4* in colorectal cancer and investigated a possible tumor suppressor function of NDRG4 by measuring colony formation and cell proliferation, migration, and invasion in colorectal cancer cell lines that stably overexpressed *NDRG4*. Finally, we investigated the potential utility of *NDRG4* promoter methylation as a biomarker for early detection of colorectal cancer in stool.

Materials and Methods

Study Population and Tissue Samples

NDRG4 promoter methylation was investigated in two independent well-characterized tissue series from colorectal carcinoma patients, adenoma patients, and control subjects without cancer. The first hospital-based series consisted of formalin-fixed, paraffin-

CONTEXT AND CAVEATS

Prior knowledge

Identification of tumor suppressor gene promoter hypermethylation in fecal DNA is a promising strategy for noninvasive detection of colorectal cancer. N-Myc downstream-regulated gene 4 (*NDRG4*) is a potential tumor suppressor in colorectal cancer.

Study design

NDRG4 promoter methylation and expression were analyzed in human colorectal cancer cell lines, noncancerous colon mucosa, and colorectal cancer tissue. NDRG4 tumor suppressor functions were examined in colorectal cancer cells. *NDRG4* promoter methylation was examined as a potential biomarker in stool from colorectal cancer patients and subjects without colorectal cancer.

Contribution

NDRG4 promoter methylation was prevalent in colorectal cancers compared with noncancerous colon mucosa. NDRG4 mRNA and protein expression were decreased in colorectal cancer tissue compared with noncancerous colon mucosa. *NDRG4* overexpression in human colorectal cancer cells inhibited colony formation and cell proliferation and invasion in vitro. A methylation-specific polymerase chain reaction assay for *NDRG4* promoter methylation identified colorectal cancer when it was present (sensitivity) in 53% of colorectal cancer cases and correctly categorized a subject as cancer free (specificity) 100% of the time.

Implications

NDRG4 is a candidate tumor suppressor gene in colorectal cancer. *NDRG4* promoter methylation is a potential biomarker for the noninvasive detection of colorectal cancer in stool samples.

Limitations

Not all stool samples from colorectal cancer patients were collected before colonoscopy as was done for the control subjects. The colorectal cancer patients were older than the subjects without colorectal cancer.

From the Editors

embedded colorectal cancer tissues (n = 90) from patients who were older than 50 years at colorectal cancer diagnosis during 1995–2003 and were retrospectively retrieved from the tissue archive of the Department of Pathology of the Maastricht University Medical Center. We also retrieved noncancerous healthy colon mucosa (n = 79) and adenoma (n = 62) tissues from these patients when available. As control tissue, we used histologically normal biopsy material from control subjects who underwent endoscopy during 1987–2004 for nonspecific abdominal complaints (n = 51), adenoma biopsy samples from patients diagnosed during 1988–1995 and who did not develop colorectal cancer within 10 years of the adenoma diagnosis (n = 22), and resected colon mucosa from patients diagnosed during 1985–2004 with various inflammatory bowel conditions (n = 33). The inflammatory bowel conditions in the latter group of control tissues included Crohn disease (n = 1), colitis ulcerosa (n = 5), nonspecific inflammation (n = 9), and diverticulitis (n = 18). Control tissues were excluded if the patient had been diagnosed with colorectal cancer in the past or during follow-up. We excluded patients with and without colorectal cancer who had been diagnosed with additional cancers (excluding nonmelanoma skin cancer). Characteristics of the study populations are shown in Supplementary

Figure 1, Supplementary Table 1, and Supplementary Table 2 (available online). Numbers of samples in the results section may differ from those indicated in the table because not all samples could be amplified by using methylation-specific PCR.

The second population-based series of formalin-fixed, paraffin-embedded colorectal cancers ($n = 184$) was randomly selected from the prospective Netherlands Cohort Study on Diet and Cancer (NLCS), which has been described in detail elsewhere (17,18). The 184 patients from whom this series of colorectal cancers were obtained were similar to the complete group of eligible colorectal cancer patients in the NLCS with respect to age at diagnosis, sex, TNM stage (19), and tumor location. This study was approved by the Medical Ethical Committee (MEC) of the Maastricht University Medical Center.

DNA Isolation

A 5- μm section of each tissue block was stained with hematoxylin and eosin and reviewed by the study pathologist (A. P. de Bruïne). Five sections (20 μm thick) were deparaffinized and subjected to genomic DNA extraction by using a Puregene DNA isolation kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Collection and Preparation of Fecal DNA

Stool samples were obtained from healthy colonoscopy-negative control subjects older than 50 years who underwent colonoscopy screening for colorectal cancer within the framework of a workplace-based community colorectal cancer study at the Maastricht University Medical Center. Stool samples and colorectal cancer tissues were collected from colonoscopy-confirmed colorectal cancer case patients who were diagnosed with all stages of colorectal cancer at the VU University Medical Center in Amsterdam. Two independent sets of noncancerous control subjects and colorectal cancer patients were included in this study: a training set consisting of 28 colorectal cancer patients and 45 noncancerous control subjects and a test set consisting of 47 colorectal cancer patients and 30 noncancerous control subjects. Series characteristics are shown in Supplementary Table 3 (available online). The MEC of the Maastricht University Medical Center and the Dutch Health Council approved this study. Written informed consent was obtained from all subjects who provided stool samples. All control stool samples from both sets of patients, one colorectal cancer stool sample from the training set, and three colorectal cancer stool samples from the test set were collected within 2 weeks before colonic purgation and colonoscopy. Twenty-seven colorectal cancer stool samples from the training and 44 colorectal cancer stool samples from the test set were collected 5–7 days after colonoscopy and before resection of the tumor.

Stool stabilization buffer was added to the stool sample by the subject immediately after defecation (EXACT Sciences, Marlborough, MA), and stool samples were processed within 48 hours after defecation. For recovery of human DNA, whole-stool samples were homogenized in a sevenfold excess volume of stool stabilization buffer and aliquoted in 32-mL portions that contained the equivalent of 4 g of stool each. Single aliquots were centrifuged at 13 100g for 2 minutes, and the supernatants were incubated with RNase A (80 U/mL) for 60 minutes at 37°C. Total DNA was precipitated by using 2.2 mL of 3 M sodium acetate

(pH 5.2) and 22 mL of 100% isopropanol, centrifuged at 4500g for 5 minutes, and resuspended in 4 mL of 1 mM EDTA, 0.01 M Tris-HCl (pH 7.4).

Half of each DNA sample was stored at -20°C , and the other half was purified as follows. Stool lysis buffer (1.5 mL; ASL buffer; Qiagen) and an InhibitEX tablet (Qiagen) were added to 2 mL of the DNA sample, and the mixture was centrifuged at 4500g for 5 minutes. After centrifugation, the supernatant was pipetted into a new tube and the pellet was discarded. We added 2 mL of the supernatant to 150 μL of proteinase K (>600 mAU/mL; Qiagen), then added 2.4 mL of lysis buffer (AL buffer; Qiagen), and incubated the mixture for 10 minutes at 70°C. We next added 2 mL of ethanol to the incubated sample, and the mixture was loaded onto a QiAamp Midi column (Qiagen), which was centrifuged at 1850g for 3 minutes. The column was washed sequentially with 2 mL of wash buffer 1 (AW1 buffer; Qiagen) and 2 mL of wash buffer 2 (AW2 buffer; Qiagen), with centrifugation at 4500g for 15 minutes. We added 200 μL of elution buffer (buffer AE; Qiagen) onto the membrane of the column, and the column was incubated at room temperature for 5 minutes. Finally, the column was centrifuged at 4500g for 2 minutes. The eluted fecal DNA (2 μg) was subjected to bisulfite modification in 96-well plates (Tecan, Männedorf, Switzerland) by using an EZ-96 DNA Methylation kit (Zymo Research Co, Orange, CA) according to the manufacturer's protocol. Bisulfite-treated fecal DNA was concentrated by using a DNA Clean & Concentrator kit (Zymo Research Co). *NDRG4* promoter methylation in fecal DNA was analyzed by quantitative methylation-specific PCR as described below.

Sodium Bisulfite Conversion, Sequencing, and Quantitative Methylation-Specific PCR

Sodium bisulfite modification, which converts unmethylated cytosine residues to uracil residues, was carried out on 500 ng genomic DNA isolated from the tissue sections and colorectal cancer cell lines (HT29, SW48, CaCo2, Colo205, RKO, LS174T, HCT116, and SW480) with the use of an EZ DNA methylation kit (Zymo Research Co) according to the manufacturer's instructions. *NDRG4* methylation-specific PCR analysis was performed on bisulfite-modified DNA as described in detail elsewhere (20,21).

For sequencing, bisulfite-modified DNA was amplified using methylation-specific primers (shown in Supplementary Table 3, available online) and a PCR profile consisting of an initial denaturation at 95°C for 5 minutes; followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 60°C, and 1 minute at 72°C; and a final extension at 72°C for 5 minutes. PCR products were cloned by using a TOPO-TA cloning kit (Invitrogen, Breda, the Netherlands), and six independent bacterial clones were sequenced by using an automated DNA sequencer (Applied Biosystems, Foster City, CA). Quantitative methylation-specific PCR was performed by using a 7900HT real-time PCR system (Applied Biosystems) as follows: 2.4 μL bisulfite-modified DNA was added to a PCR mix containing buffer [16.6 mM $(\text{NH}_4)_2\text{SO}_4$, 67 mM Tris, 6.7 mM MgCl_2 , 10 mM β -mercaptoethanol], dATP, dCTP, dGTP, and dTTP (each at 5 mM), forward primer (6 ng/ μL), reverse primer (18 ng/ μL), a single-stranded oligonucleotide hybridization probe (0.16 μM), bovine serum albumin (BSA; 0.1 μg), and Jumpstart *Taq* polymerase (0.4 U; Sigma-Aldrich). β -Actin was used as a reference gene for normalization.

The PCR program was as follows: 5 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 (20–2 000 000 copies) 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 (Ct value) to the corresponding plasmid copy. Primer sequences are provided in Supplementary Table 3 (available online). Samples were handled and analyzed in a blinded fashion during storage, DNA isolation, and PCR analysis. One quantitative methylation-specific PCR experiment was performed for each independent set of patients.

Cell Culture and Transfections

Human colorectal cancer cell lines (HT29, SW48, CaCo2, Colo205, RKO, LS174T, HCT116, and SW480; all from LGC, Teddington, UK) were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone, Etten-Leur, the Netherlands). To investigate the effect of reexpression of *NDRG4*, RKO and HCT116 cells were treated for 3 days with 1 μ M DAC (Sigma). The full-length *NDRG4* cDNA (Origene, Rockville, MD) was subcloned into a pCMV6-Neo vector (Origene) to create pCMV6-NDRG4. HCT116 cells were transfected with pCMV6-NDRG4 or empty vector (pCMV6) by use of a Nucleofector Kit V (Amaxa Biosystems, Gaithersburg, MD) according to the manufacturer's guidelines. RKO cells were transfected with pCMV6-NDRG4 or pCMV6 by using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's protocol. Transfected HCT116 and RKO cells were grown for 10 days in medium containing G418 (at 400 μ g/mL for HCT116 and 1 mg/mL for RKO; Invitrogen) to select for cells that were stably transfected with the pCMV6-based plasmids.

Quantitative Real-Time PCR

Total RNA was isolated from colorectal cancer cell lines and tissues from patients and control subjects by using an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions and treated with RNase-free DNase (Qiagen) to remove contaminating genomic DNA. cDNA was synthesized from 1 μ g of the DNase-treated RNA by using an Iscript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative real-time PCR to quantify *NDRG4* mRNA levels was performed by using SYBR Green PCR master mix (Applied Biosystems, Nieuwekerk a/d IJssel, the Netherlands) as described previously (22). Cyclophilin A was used as a reference gene for normalization. Primers used are listed in Supplementary Table 3 (available online).

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections (5 μ m thick). Sections were deparaffinized in xylene, rehydrated, and incubated with 0.3% hydrogen peroxide in methanol for 30 minutes. The sections were incubated with Tris-buffered saline (TBS), 20% FCS, and 0.1% Tween to block nonspecific antibody binding, followed by incubation with the anti-*NDRG4* monoclonal antibody (Abnova Corporation, Taipei City, Taiwan) diluted 1:6000 in TBS with 0.1% Tween and 0.5% BSA. Sections were incubated with a

horseradish peroxidase-conjugated secondary antibody against mouse, rabbit, and rat IgGs (Poly-HRP GAM/R/R IgG) (Immunovision Technologies, Burlingame, CA), and bound antibody was visualized by using 3,3'-diaminobenzidine substrate as a chromogen (Dako, Glostrup, Denmark) followed by hematoxylin counterstaining.

Colony Formation Assay

Colorectal cancer RKO and HCT116 cells were transfected in six-well plates (1 \times 10⁶ cells per well) with pCMV6 or pCMV6-NDRG4 as described above. The next day, the cells were diluted 1:20 and G418 (at 1 mg/mL for RKO 400 μ g/mL for HCT116) was added to the medium to select for cells in which the plasmids had stably integrated into genomic DNA. After 14 days of selection, colonies were stained by using Giemsa's azur eosin methylene blue solution (Merck, Darmstadt, Germany) and counted. Colony formation was assessed in four different experiments (two replicate wells per experiment).

In Vitro Cell Proliferation, Migration, and Invasion Assays

HCT116 cells were seeded onto 96-well plates (5000 cells per well), and cell numbers were counted 24, 48, 72, and 96 hours later (three wells per time point). In addition, after 96 hours of incubation, the cultures were pulse labeled for 6 hours with [methyl-³H] thymidine (0.3 μ Ci per well; Amersham Life Science, Roosendaal, the Netherlands). Cells were harvested by using a cell harvester, and [³H]thymidine activity was measured by using a liquid scintillation counter. Three independent experiments were performed (three replicate wells per experiment).

Cell migration and invasion assays were performed using matrigel-coated (invasion assay) or uncoated (migration assay) 24-well transwell plates (8- μ m pore size) (BD Biosciences, Franklin Lakes, NJ). Briefly, 2 \times 10⁵ HCT116 cells in DMEM containing 1% FCS were seeded into the upper chamber of each well, and DMEM containing 20% FCS was placed in the lower chamber. After 48 hours of incubation, the transwells were disassembled and the membranes that separated the upper and lower chamber of each transwell were fixed with methanol and stained with 1% toluidine blue in 1% borax and the cells on the lower surface of the membrane were counted with the use of a light microscope. Transwell experiments were assessed in three different experiments (two replicate wells per experiment).

Statistical Analysis

For comparison between *NDRG4* methylation frequencies in normal, adenoma, and carcinoma tissues from colorectal cancer patients and normal and adenoma tissues from control subjects without colorectal cancer, we used logistic regression (Table 1). Because we observed statistically significant differences in age between the cancer patients and control subjects (analyzed using one-way analysis of variance [ANOVA]) and in tumor location among the cancer patients (analyzed using Pearson χ^2 test) (Supplementary Table 1, available online), logistic regression analyses were adjusted for age and location. To compare the prevalence of *NDRG4* promoter methylation in colorectal cancer tissue in relation to clinicopathological features, the Pearson χ^2 test (TNM stage, tumor location, and sex) or Fisher exact test

Table 1. *NDRG4* promoter methylation frequencies in normal, adenoma, and carcinoma tissues from CRC patients and normal and adenoma tissues from control subjects without CRC*

| MSP primer | Carcinoma from CRC patients | Normal tissue from control subjects | P | Normal tissue | | P | Adenoma tissue | | P |
|------------|-----------------------------|-------------------------------------|-------|---------------|------------------|------|----------------|------------------|------|
| | | | | CRC patients | Control subjects | | CRC patients | Control subjects | |
| NDRG4 p1 | 71/83 (86) | 2/48 (4) | <.001 | 9/78 (12) | 2/48 (4) | >.99 | 41/62 (66%) | 12/22 (55) | >.99 |
| NDRG4 p2 | 55/77 (71) | 0/28 (0) | <.001 | 2/80 (3) | 0/28 (0) | >.99 | 24/58 (41%) | 4/31 (13) | >.99 |

* Methylation frequency is presented as the number of methylated samples divided by the total number of samples analyzed (%). *P* values (two-sided) were generated by using logistic regression with adjustment for age (continuous data) and tumor location (categories: proximal and distal location). *P* values are Bonferroni adjusted, and the cutoff for statistical significance is *P* = .017. CRC = colorectal cancer; *NDRG4* = N-Myc downstream-regulated gene 4; MSP = methylation-specific polymerase chain reaction; p1 = methylation-specific primer pair 1; p2 = methylation-specific primer pair 2.

(age at diagnoses) was used (Table 2). In the hospital-based series, noncancerous control tissues, adenoma tissues, and carcinoma tissues were obtained from the same patients. These paired samples were analyzed by using the McNemar test to compare *NDRG4* methylation frequencies in carcinoma, adenoma, and normal tissues from colorectal cancer patients (Table 3). Where appropriate, the Bonferroni method was used to correct for multiple comparisons.

For quantitative methylation-specific PCR analysis, we used receiver operating characteristic (ROC) curve analysis and the area under the curve (AUC) to determine the best cutoff value for highest sensitivity and specificity. *NDRG4* promoter methylation was considered positive if the methylation value was greater than the cutoff. Because age differences were expected between cancer patients and control subjects, an ROC curve and generalized linear (ROC-GLM) regression model was used to assess the

influence of the age difference on the accuracy of *NDRG4* methylation as a biomarker for the detection of colorectal cancer.

Analysis of cell growth curves was performed by means of two-way ANOVA. The Student *t* test was used for analyses of [³H]thymidine incorporation and anchorage-independent cell growth. The Mann–Whitney rank sum test was used to analyze data obtained in the colony formation, quantitative real-time PCR, migration, and invasion assays.

All *P* values are two-sided, and *P* values less than or equal to .05 were considered statistically significant. Data analysis was done by using SPSS software (version 12.0.1; SPSS Inc., Chicago, IL)

Results

NDRG4 Promoter Methylation and mRNA Expression in Colorectal Cancer Cell Lines

The promoter region of the *NDRG4* gene (National Center for Biotechnology Information [NCBI] accession number NM_020465) contains a dense CpG island located from nucleotides –556 to +869 relative to the transcription start site (Figure 1, A). To assay this region for potential methylation, we examined eight human colorectal cancer cell lines by methylation-specific PCR using primers located from –250 to +10 relative to the transcription start site (primers are listed in Supplementary Table 3, available online). The *NDRG4* promoter was methylated in all of the cell lines except SW480 (Figure 1, B). To investigate the pattern of CpG island methylation in the *NDRG4* promoter, we sequenced sodium bisulfite–modified genomic DNA isolated from HCT116 (*NDRG4* promoter methylation positive) and SW480 (*NDRG4* promoter methylation negative) cells. The promoter region spanning 39 CpG sites (–251 to +10) was PCR amplified using sodium bisulfite–modified genomic DNA as template. Bisulfite sequencing confirmed the methylation-specific PCR data in that HCT116 cells showed almost complete methylation, whereas SW480 cells showed almost no methylated CpGs (Figure 1, C). To investigate whether promoter methylation was associated with inhibition of gene expression, we measured *NDRG4* mRNA levels in HCT116 and RKO cells incubated with and without the DNA methylation inhibitor DAC. In both cell lines, endogenous *NDRG4* mRNA levels were statistically significantly higher in DAC-treated cells than in untreated cells (DAC treated vs untreated, RKO cells: 4.4-fold increase, 95% confidence interval [CI] = 3.17- to 5.63-fold increase, *P* = .014; HCT116 cells: 1.7-fold increase, 95% CI = 0.93- to 2.47-fold increase, *P* = .037) (Figure 1, D).

Table 2. Prevalence of *NDRG4* promoter methylation in colorectal cancer tissue in relation to clinicopathological features for two independent series*

| Characteristic | Hospital-based series† | Population-based series‡ |
|----------------------------|------------------------|--------------------------|
| TNM stages§ | | |
| I | 11/12 (92) | 30/42 (71) |
| II | 23/28 (82) | 42/57 (74) |
| III | 29/32 (91) | 39/56 (70) |
| IV | 8/11 (72) | 17/21 (81) |
| <i>P</i> | .431 | .790 |
| Tumor location | | |
| Proximal | 34/39 (87) | 47/58 (81) |
| Distal | 37/42 (89) | 81/118 (69) |
| <i>P</i> | 1.00 | .141 |
| Sex | | |
| Male | 34/41 (83) | 71/95 (75) |
| Female | 37/42 (88) | 57/81 (70) |
| <i>P</i> | .548 | .611 |
| Age at diagnosis, y | | |
| ≤70 | 30/32 (94) | 83/117 (71) |
| >70 | 41/51 (80) | 45/59 (76) |
| <i>P</i> | .117 | .453 |

* Methylation frequency is presented as the number of methylated samples divided by the total number of samples analyzed (%). *P* values are from two-sided χ^2 tests. *NDRG4* = N-Myc downstream-regulated gene 4.

† Collected from the tissue archive of the Department of Pathology of the University Hospital Maastricht.

‡ Prospective Netherlands Cohort Study on Diet and Cancer.

§ Reference 23.

Table 3. *NDRG4* promoter methylation frequencies in carcinoma, adenoma, and normal tissues from colorectal cancer patients*

| MSP primer | Normal tissue | Adenoma tissue | <i>P</i> | Normal tissue | Carcinoma tissue | <i>P</i> | Adenoma tissue | Carcinoma tissue | <i>P</i> |
|-----------------|---------------|----------------|----------|---------------|------------------|----------|----------------|------------------|----------|
| <i>NDRG4</i> p1 | 5/36 (14) | 22/36 (61) | <.001 | 5/31 (16) | 26/31 (84) | <.001 | 20/32 (63) | 26/32 (81) | .540 |
| <i>NDRG4</i> p2 | 0/32 (0) | 11/32 (34) | .003 | 0/30 (0) | 22/30 (73) | <.001 | 13/33 (39) | 25/33 (76) | .012 |

* Methylation frequency is presented as the number of methylated samples divided by the total number of samples analyzed (%). Frequencies for some types of tissue vary because for some patients we did not have simultaneous information on both types of tissue in the analysis (eg, not all patients with adenomas also had both normal and carcinoma tissue available). *P* values (Bonferroni adjusted) are from a two-sided McNemar test; the cutoff for statistical significance is *P* = .017. *NDRG4* = N-Myc downstream-regulated gene 4; MSP = methylation-specific polymerase chain reaction.

Prevalence of *NDRG4* Promoter Methylation in Primary Colorectal Adenomas and Carcinomas

Sequence analysis of sodium bisulfite-modified genomic DNA isolated from three pairs of primary colorectal cancer tissues and matched noncancerous colon normal mucosa showed dense methylation at the *NDRG4* promoter (region -251 to +10 relative to the transcription start site) in the colorectal cancers but almost no *NDRG4* promoter methylation in matched normal colon mucosa (Figure 2, A). It is interesting that the density of methylation was higher in the upstream region (ie, the region more 5' relative to the

transcription start site) of the *NDRG4* CpG island than in the more downstream region (Figure 2, A).

To examine the methylation status of the *NDRG4* promoter in a large series of noncancerous colon mucosa, adenoma, and colorectal cancer tissues, we performed methylation-specific PCR with primer pair 1 (which is situated more to the 5'-end of the *NDRG4* promoter region, where the *NDRG4* methylation density was higher compared with the upstream region; Figure 1, A). The frequency of *NDRG4* promoter methylation was lower in the normal mucosa from the control subjects than in the colorectal cancer

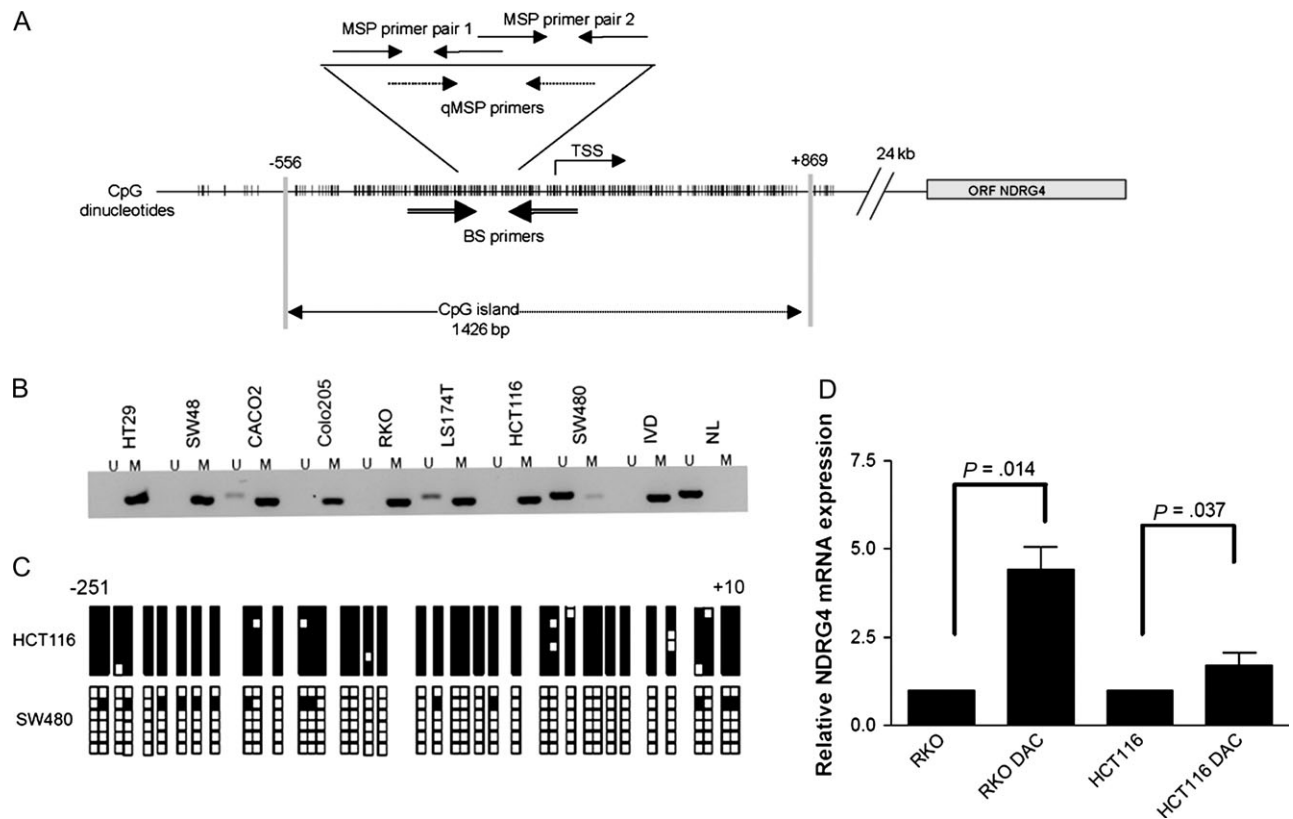


Figure 1. N-Myc downstream-regulated gene 4 (*NDRG4*) promoter structure, promoter methylation, and mRNA expression in colorectal cancer cell lines. **A)** Schematic representation of the promoter region of *NDRG4* (NM_020465). A dense CpG island is located between nucleotides -556 and +869 relative to the transcription start site (TSS). **Vertical lines** represent the locations of CpG dinucleotides, the **gray rectangle** indicates the open reading frame (ORF) of *NDRG4*, and **paired arrows** indicate the locations of the amplicons identified by methylation-specific polymerase chain reaction (MSP), quantitative MSP (qMSP), and bisulfite sequencing (BS) primers. **B)** Electrophoretic analysis of MSP amplification products in eight colorectal cancer cell lines. U = unmethylated, M = methylated, IVD = in vitro methylated

DNA, NL = normal lymphocytes. **C)** Bisulfite sequencing of colorectal cancer HCT116 and SW480s. Six different bacterial clones were sequenced. Each **row** represents an individual cloned allele that was sequenced following sodium bisulfite DNA modification. Each **box** indicates a CpG dinucleotide (**black box** = methylated CpG site; **white box** = unmethylated CpG site). **D)** *NDRG4* mRNA expression in colorectal cancer RKO and HCT116 cells with and without treatment with the DNA methylation inhibitor 5-aza-2'-doxycytidine (DAC). Quantification is presented as mean values (**error bars** correspond to 95% confidence intervals) relative to untreated cells from three independent experiments (normalization was against cyclophilin A). Statistical analysis was done using the Mann-Whitney rank sum test (two-sided).

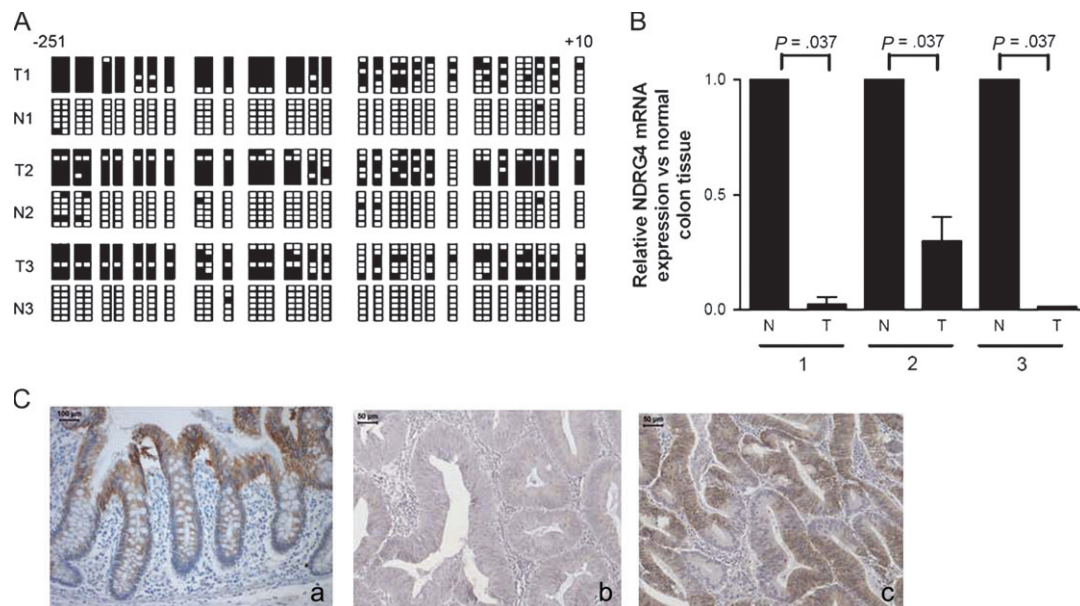


Figure 2. N-Myc downstream-regulated gene 4 (*NDRG4*) promoter methylation and expression in primary colorectal cancer. **A)** Bisulfite sequencing of colorectal cancer (T1–3) and the matched normal colon mucosa (N1–3) from three colorectal cancer patients. Six different bacterial clones per tissue sample were sequenced. Each row represents an individual cloned allele that was sequenced following sodium bisulfite DNA modification. Each box indicates a CpG dinucleotide (black box = methylated CpG site; white box = unmethylated CpG site). **B)** *NDRG4* mRNA levels measured by real-time polymerase chain reaction in colon cancer tissues (T) and matched normal colon tissue samples (N) from the three colorectal cancer

patients in (A). For each patient, the level of *NDRG4* mRNA expression in normal mucosa tissue was set to equal 1. Data are presented as mean values relative to normal mucosa tissue of three independent experiments (error bars correspond to 95% confidence intervals). Statistical analysis was performed using the Mann–Whitney rank sum test (two-sided). **C)** Immunohistochemical localization of *NDRG4* protein expression in normal colon mucosa (a, $\times 10$ magnification) and colorectal cancer (b, c, $\times 40$ magnification). Blue (hematoxylin) staining represents the nuclear staining and brown (DAB) staining represents the antibody (*NDRG4*). Each image is from a different patient.

tissue from the colorectal cancer patients (2/48 [4%] vs 71/83 [86%]; $P < .001$) (Table 1). The frequency of *NDRG4* promoter methylation in adjacent normal mucosa of colorectal cancer patients did not differ statistically significantly from that in the normal mucosa of control subjects (9/78 [12%] vs 2/48 [4%]; $P > .99$) (Table 1). Little or no *NDRG4* promoter methylation was found in skin, renal cell, ovarian, prostate, breast, or esophageal squamous cell carcinomas (data not shown). By contrast, the *NDRG4* promoter was frequently methylated in adenocarcinomas of the esophagus (13/16 [81%]) and in diffuse-type (8/11 [73%]) and intestinal-type (9/11 [82%]) adenocarcinomas of the stomach (data not shown). We also compared the frequency of *NDRG4* promoter methylation in premalignant lesions from patients with and without colorectal cancer. We observed no statistically significant difference in the frequency of *NDRG4* promoter methylation between adenomas obtained from colorectal cancer patients that developed synchronously or metachronously to the tumor and adenomas obtained from patients that did not develop colorectal cancer after 10 years of follow-up (41/62 [66%] vs 12/22 [55%]; $P > .99$) (Table 1).

To confirm the high prevalence of *NDRG4* promoter methylation in colorectal cancer, we analyzed a second independent, population-based series of colorectal cancers and observed that *NDRG4* promoter methylation was present in 70% (128/184) of colorectal cancer patients (data not shown). In each of the two independent series of colorectal cancer patients, *NDRG4* promoter methylation was not associated with age at diagnosis (mean age = 70 years), sex, proximal vs distal tumor location, or TNM stage (Table 2).

We next investigated whether *NDRG4* promoter methylation changes during colorectal cancer progression by comparing the frequency of *NDRG4* promoter methylation in samples of normal mucosa, adenoma, and colorectal cancer tissues (Table 3). The *NDRG4* promoter was more frequently methylated in colorectal carcinomas than in matched normal mucosa adjacent to the tumor (26/31 [84%] vs 5/31 [16%]; $P < .001$) (Table 3). Adenoma samples from colorectal cancer patients also had a statistically significantly higher *NDRG4* promoter methylation frequency than normal colon samples (22/36 [61%] vs 5/36 [14%]; $P < .001$) (Table 3). Finally, the frequency of *NDRG4* promoter methylation was higher in colorectal carcinomas than in matched adenoma samples, but the difference was not statistically significant (26/32 [81%] vs 20/32 [63%]; $P = .54$) (Table 3).

Heterogeneity of *NDRG4* Promoter Methylation

As described above, we observed that the density of methylation was higher in the upstream region of the *NDRG4* promoter CpG island than in the more downstream region. We therefore used methylation-specific PCR primer pair 2, which amplifies a region downstream of primer pair 1 (Figure 1, A), to investigate this region for *NDRG4* promoter methylation. The sensitivity for colorectal cancers (the proportion of people with disease who have a positive test result) decreased from 86% with primer pair 1 to 71% with primer pair 2, whereas the specificity (the proportion of people without disease with a negative test result) increased from 96% to 100%. Intriguingly, using primer pair 2, we found a statistically significant difference in the frequency of *NDRG4* promoter

methylation between matched adenomas and carcinomas from the colorectal cancer patients (13/33 [39%] vs 25/33 [76%]; $P = .012$), which was not observed using primer pair 1 (Table 3).

NDRG4 mRNA and Protein Expression in Colorectal Cancer

We next examined whether methylation of the CpG island in the *NDRG4* promoter is associated with gene silencing by investigating *NDRG4* mRNA expression in colorectal cancer tissue and matched normal colon mucosa from three colorectal cancer patients. In each of the three pairs of tissue, the *NDRG4* mRNA level in the colorectal cancer was statistically significantly lower than that in the matched normal colon mucosa ($P = .037$ for all three matched pairs). Compared with the *NDRG4* mRNA level in the matched normal tissue (set at 100%), the expression of *NDRG4* in tumor 1, 2, and 3 was 3% (95% CI = -3% to 8%), 31% (95% CI = 9% to 58%), and 1.5% (95% CI = 1.3% to 1.7%), respectively (Figure 2, B).

Next, we performed immunohistochemistry to investigate *NDRG4* protein expression in a matched pair of normal colonic mucosa and colorectal cancer tissue from a colorectal cancer patient. In normal colon mucosa, *NDRG4* expression was predominantly in basolateral membranes within colonocytes and increased in intensity near the mucosal surface (Figure 2, C, panel a). The matched colorectal cancer tissue showed heterogeneous cytoplasmic staining with the anti-*NDRG4* antibody. Similar results were observed in matched pairs of tissues from other colorectal cancer patients (data not shown). In most tumors, less than 50% of the tumor area showed weak to focally strong *NDRG4* expression (Figure 2, C, panels b and c).

To investigate the association between *NDRG4* promoter methylation and *NDRG4* expression, we performed immunohistochemical analysis of *NDRG4* protein expression on tissues from 80 colorectal cancer patients of the population-based series. We observed no association between *NDRG4* promoter methylation and *NDRG4* expression, suggesting that an alternative mechanism might account for *NDRG4* inactivation in colorectal cancer. Therefore, we analyzed macrodissected colorectal cancer tissue and matched normal tissues from 86 colorectal cancer case patients of the population-based series for loss of heterozygosity. In addition, 12 primary colorectal cancers and the colorectal cancer cell lines HCT116 and SW480 were analyzed for *NDRG4* mutations. We observed loss of heterozygosity in 27 (31%) of 86 colorectal cancers. No inactivating mutations within the coding region of the *NDRG4* gene were detected in the 12 colorectal carcinomas. However, we found one novel nonsynonymous mutation in the SW480 cell line (40662A→AG Ile65Val [an A-to-G substitution at nucleotide 4066, resulting in an isoleucine-to-valine substitution at amino acid 65]). In addition, two previously reported single-nucleotide polymorphisms (SNPs) were detected among the 12 colorectal cancers. One SNP was observed in one of the 12 colorectal cancers (43760G→GG Val224Val [a G-to-GG substitution at nucleotide 43760, no difference in amino acid]); NCBI SNP database [dbSNP] accession number rs17821543). The second SNP was observed in nine of the 12 colorectal cancers (48311A→AG Ser354Ser [an A-to-AG substitution at nucleotide 43760, no difference in amino acid]); NCBI dbSNP accession number rs42945).

Effect of NDRG4 Overexpression in Colorectal Cancer Cell Lines

To examine whether *NDRG4* acts as a tumor suppressor in colorectal cancer cells, we characterized HCT116 and RKO cells that were transfected with an expression vector harboring the full-length *NDRG4* cDNA or empty vector (control). *NDRG4* transfectants (pools of stably transfected cells) showed increased expression of *NDRG4* mRNA compared with control cells transfected with empty vector (HCT116 52%, $P = .005$; RKO 69%, $P = .037$). In addition, *NDRG4* protein expression was increased in the *NDRG4* transfectants compared with the control cells transfected with empty vector (data not shown). Compared with control transfectants, transfection with *NDRG4* statistically significantly reduced the number of G418-resistant colonies (HCT116: 77% reduction [95% CI = 66% to 90% reduction], $P = .014$; RKO: 69% reduction [95% CI = 61% to 76% reduction], $P = .014$) (Figure 3, C and D) and statistically significantly decreased cell proliferation as measured by [³H] thymidine activity (HCT116: 40% reduction [95% CI = 27% to 44% reduction], $P < .001$) (Figure 3, E and F). *NDRG4* transfectants displayed reduced invasion through matrigel-coated transwell membranes compared with control transfectants (HCT116: 48% reduction [95% CI = 31% to 64% reduction], $P < .001$) (Figure 3, G). However, transfection of *NDRG4* had no effect on HCT116 cell migration compared with control-transfected cells (Figure 3, H). Taken together, these data suggest that *NDRG4* exhibits tumor-suppressive effects in human colorectal cancer cells.

Sensitivity and Specificity of NDRG4 Promoter Methylation in Fecal DNA for the Detection of Colorectal Cancer

The high prevalence of *NDRG4* promoter methylation in colorectal cancer and the absence of methylation in normal colon mucosa suggested that *NDRG4* promoter methylation could be a sensitive and specific biomarker for the noninvasive detection of colorectal cancer in human stool. Therefore, we developed a quantitative molecular beacon-based methylation-specific PCR assay that used a primer pair situated between methylation-specific PCR primer pair 1 and methylation-specific PCR primer pair 2 and fecal DNA isolated from stool (Figure 1, A). We first examined *NDRG4* promoter methylation as a biomarker for colorectal cancer in a training set comprising 28 colorectal cancer patients and 45 healthy control subjects. We used the data from the training set to construct an ROC curve with an AUC of 0.77 (95% CI = 0.66 to 0.86) (Figure 4). Using a cutoff level of 1.22 copies, which gave the highest sensitivity and specificity for the detection of colorectal cancer, we detected *NDRG4* promoter methylation in 17 of the 28 colorectal cancer patients, yielding a sensitivity of 61% (95% CI = 43% to 79%) for the detection of colorectal cancer. Three (7%) of the 45 healthy control subjects tested positive for *NDRG4* methylation, which resulted in a specificity of the assay of 93% (95% CI = 90% to 97%). To test the accuracy of the *NDRG4* promoter methylation cutoff generated from the training set, we assayed *NDRG4* promoter methylation by using quantitative methylation-specific PCR in an independent series of 47 colorectal cancer patients

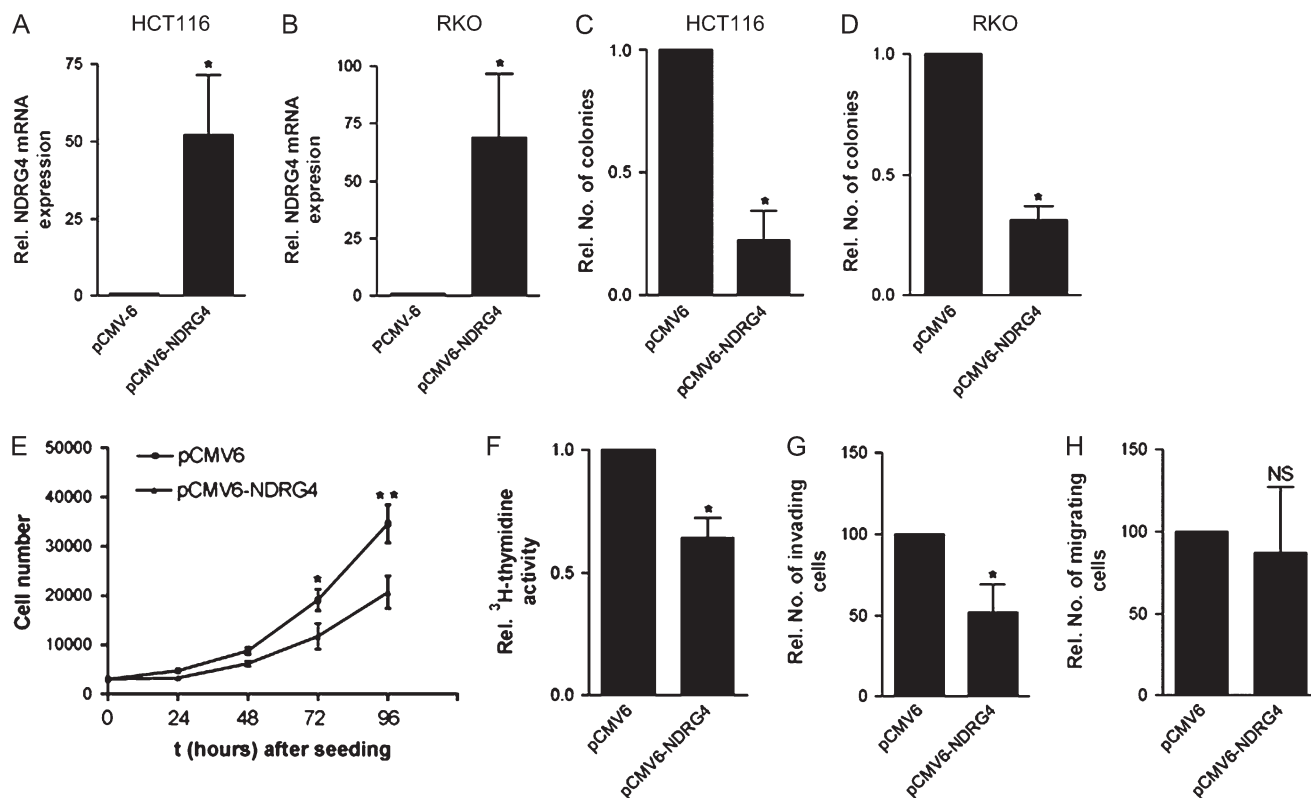


Figure 3. Functional assays of N-Mye downstream-regulated gene 4 (*NDRG4*) in colorectal cancer. (A, B) *NDRG4* mRNA expression measured by real-time polymerase chain reaction in HCT116 (A) and RKO (B) cells stably transfected with *NDRG4* expression vector (pCMV6-*NDRG4*) or empty vector (pCMV6). Results are plotted as mean values of mRNA expression relative to control vector in five (HCT116) and three (RKO) independent experiments (HCT116; * $P = .005$, RKO; * $P = .037$). (C, D) Colony formation by HCT116 (C) and RKO (D) cells transfected with pCMV6 or pCMV6-*NDRG4* and grown for 2 weeks in medium containing G418. Results are plotted as the mean colony numbers relative to pCMV6 transfectants in four independent experiments (C and D: * $P = .014$). Statistical analysis in panels A–D was performed using the Mann–Whitney rank sum test (two-sided). (E) Cell proliferation assay. *NDRG4*-transfected HCT116 cells (pCMV6-*NDRG4*) were compared with control cells transfected with empty vector (pCMV6). Results are plotted as the mean cell number in three independent experiments

(72 hours: * $P = .05$; 96 hours: ** $P < .001$; two-way analysis of variance). (F) Cell proliferation measured by ³H-thymidine incorporation. Data are expressed as mean number of proliferating *NDRG4*-transfected cells relative to control cells transfected with empty vector (pCMV6). Three independent experiments were performed (three replicate wells per experiment) (* $P < .001$; two-sided Student *t* test). (G) Invasion of HCT116 cells through matrigel-coated transwells. Results represent mean number of *NDRG4*-transfected cells that passed through the matrigel-coated membranes of the transwell relative to control cells transfected with empty vector in three independent experiments (* $P < .001$, two-sided Mann–Whitney rank sum test). (H) Migration assay. Plotted are the mean number of *NDRG4*-transfected HCT116 cells that migrated through transwell membranes not coated with matrigel relative to control cells transfected with empty vector in three independent experiments (NS = not statistically significantly different from control, Mann–Whitney rank sum test). Error bars correspond to 95% confidence intervals.

and 30 healthy control subjects, which were handled in a blinded fashion. Using the previously determined cutoff level of 1.22 methylated copies, we found that 25 of the 47 colorectal cancer patients tested positive for *NDRG4* methylation, resulting in a sensitivity of 53% (95% CI = 39% to 67%), and that none of the 30 control subjects tested positive, yielding a specificity of 100% (95% CI = 86% to 100%).

Because the mean age of the colorectal cancer patients differed statistically significantly from that of the control subjects in the training and test set (Supplementary Table 4, available online), we used an ROC-GLM regression model in the training set to assess the accuracy of *NDRG4* promoter methylation for the detection of colorectal cancer after adjustment for age. This analysis indicated that age did not statistically significantly influence the accuracy ($P = .89$, ROC-GLM regression model) (23). These data indicate that detection of *NDRG4* promoter methylation in fecal DNA can be used as a novel biomarker for detection of colorectal cancer.

Discussion

Here we describe the identification and validation of *NDRG4* promoter methylation, a novel, sensitive, and specific marker for the detection of colorectal cancer. We found a statistically significant difference in the frequency of *NDRG4* promoter methylation between colorectal cancer and normal colon mucosa. Adding inflamed mucosa, which often shows promoter CpG island methylation (24), to the normal colon mucosa control group only slightly reduced the specificity of *NDRG4* promoter methylation from 96% to 94%, indicating that *NDRG4* promoter methylation is not associated with inflammation. Although most CpG island promoter-methylated genes are frequently associated with a proximal tumor location (25), *NDRG4* promoter methylation is present in both distal colorectal cancers [often associated with chromosomal instability (26)] and proximal colorectal cancers (often associated with microsatellite instability). This finding makes *NDRG4* promoter methylation a promising marker to detect chromosomal-unstable as

well as microsatellite-unstable colorectal cancers. We also found that DNA methylation density was higher in the upstream region of the *NDRG4* promoter than in the downstream region. Therefore, we used two different pairs of methylation-specific PCR primers that amplify overlapping fragments in the CpG island to detect *NDRG4* promoter methylation. Using primer pair 1 (the one most 5' to the transcription start site) to detect *NDRG4* promoter methylation resulted in a sensitivity for colorectal cancer of 86% and for adenoma of 66% and a specificity of 96%. Using primer pair 2, the sensitivity for colorectal cancers and adenomas decreased to 71% and 41%, respectively, whereas the specificity increased to 100%. This finding suggests that *NDRG4* promoter hypermethylation initially occurs at the 5'-end of the *NDRG4* CpG island and spreads toward the transcription start site before ultimately shutting down *NDRG4* mRNA expression, as has also been observed in the promoter for *RUNX3* (27,28).

In addition, we found a statistically significantly lower methylation frequency in adenoma tissue compared with carcinoma tissue when we used primer pair 2 to detect *NDRG4* promoter methylation. This finding suggests that DNA methylation in the promoter of *NDRG4* may spread toward the transcription start site during cancer progression.

To our knowledge, expression of *NDRG4* has been documented only in brain and heart tissue by Northern blotting. Here we show that *NDRG4* mRNA and protein is expressed in normal colon tissue and that expression of both is decreased in colorectal cancer. However, we found no statistically significant association between *NDRG4* promoter methylation and *NDRG4* expression. We and others (29) also found no evidence for mutational inactivation of *NDRG4*. However, we observed loss of heterozygosity of the *NDRG4* locus at chromosome 16q in 31% of the colorectal cancers analyzed. Frequent loss of heterozygosity at 16q was previously observed in a wide variety of solid tumor types, including breast (30), liver (31,32), prostate (33), ovarian (34), and Wilms tumors (35), but, to our knowledge, has not been described in colorectal cancer. These findings suggest that promoter methylation and loss of heterozygosity contribute to the altered expression of *NDRG4* in colorectal cancer.

We also provide evidence that *NDRG4* has tumor suppressor activities in colorectal cancer. Overexpression of *NDRG4* in the colorectal cancer HCT116 cells inhibited colony formation and cell proliferation and invasion in vitro, suggesting that *NDRG4* is a tumor suppressor gene in colorectal cancer. The ability of the *NDRG4*-transfected cells to migrate in vitro was identical to that of control cells, which was also observed for *NDRG1* (4).

To investigate the potential utility of *NDRG4* promoter methylation as a noninvasive biomarker test to identify individuals who should undergo colonoscopy because they are at increased risk for colorectal cancer, we analyzed fecal DNA of colorectal cancer patients and control subjects. We showed that detection of *NDRG4* promoter methylation in fecal DNA was both sensitive and specific at identifying colorectal cancer patients in two independent series of case patients and control subjects. The prevalence of *NDRG4* promoter methylation in colorectal cancer tissue (86% and 70% in two independent series) suggests that the use of optimal protocols for the isolation of fecal DNA could, in theory,

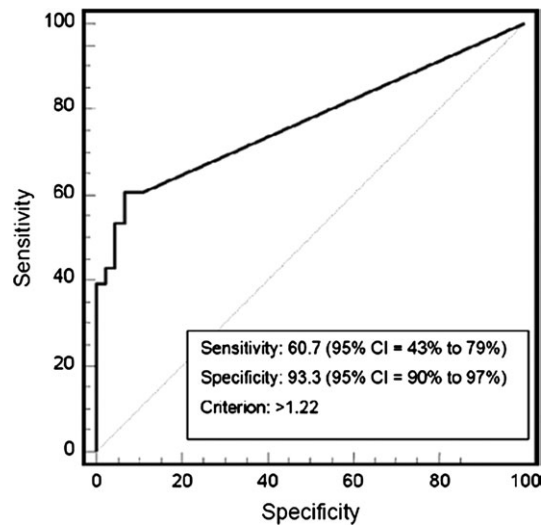


Figure 4. N-Myc downstream-regulated gene 4 (*NDRG4*) promoter methylation in fecal DNA as biomarker for colorectal cancer detection. Sensitivity and specificity at various cutoff values for the training set (which consisted of 28 colorectal cancer patients and 45 healthy control subjects) to obtain a positive test for *NDRG4* quantitative methylation-specific PCR are shown in the receiver operating characteristic (ROC) curve. The **jagged line** represents the ROC curve. The **dashed line** represents the line of no discrimination between good and bad classification. The determined optimal cutoff value for *NDRG4* promoter methylation is 1.22 copies.

yield a stool *NDRG4* quantitative methylation-specific PCR test with an even higher sensitivity. Methyl-binding domain protein columns to capture methylated DNA, which have been shown to markedly increase sensitivity without decreasing specificity (36), may be useful in this respect. Several studies have provided proof of principle for the detection of promoter CpG island hypermethylation of colorectal adenoma or colorectal cancer-derived DNA in stool (37–43) and blood (44–49). Compared with other gene promoter methylation markers described thus far (37–39, 44–48), *NDRG4* promoter methylation performs well as a novel single marker. Combining *NDRG4* promoter methylation with other DNA markers could improve its sensitivity and specificity for the detection of colorectal cancer, as has been observed for other methylation markers (50,51). In addition, the specificity of *NDRG4* promoter methylation for gastrointestinal adenocarcinomas makes it a specific biomarker for detecting gastrointestinal cancers in stool and blood.

The stool study presented here should be considered a pilot study that has specific limitations. Not all stool samples from colorectal cancer patients were collected before colonoscopy as was done for the control subjects. It is therefore possible, albeit very unlikely, that colonoscopy could have introduced artifacts in the observed methylation prevalence. In addition, the age of the colorectal cancer patients and control subjects differed statistically significantly, which raises the question of whether the detected *NDRG4* promoter methylation is associated with age. However, this possibility is highly unlikely because neither data from the two independent colorectal cancer series nor the ROC-GLM regression analysis of the stool samples showed that *NDRG4* promoter methylation was associated with age. Nevertheless, the stool data

should be validated in a large prospective screening study for colorectal cancer, as should *NDRG4* promoter methylation be combined with other methylation markers to enhance the sensitivity and/or specificity.

In conclusion, to our knowledge, this is the first study to describe a tumor suppressor role for *NDRG4* in cancer. Our data indicate that *NDRG4* promoter methylation is potentially useful as a sensitive and specific noninvasive preselection modality for identifying individuals at risk for colorectal cancer for whom colonoscopy is recommended.

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