Suppression of N-Myc Downstream-Regulated Gene 2 Is Associated with Induction of Myc in Colorectal Cancer and Correlates Closely with Differentiation

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NDRG2, a new member of the N-Myc downstream-regulated gene (NDRG) family, is a focus for study at present. Up to now, its expression and function in carcinoma remain to be elucidated. In this study, using a colorectal cancer tissue array and a series of 213 colorectal cancer samples, the relationship between Ndrg2 and c-MYC expression and tumor differentiation level was investigated. Immunohistochemistry showed that Ndrg2 expression was reduced and that c-Myc was increased in colorectal carcinomas. In addition, Ndrg2 protein levels increased from poorly differentiated to well-differentiated carcinomas ($p=0.005$). Real-time polymerase chain reaction and Western blots demonstrated quantitatively that NDRG2 mRNA and protein levels were lower in colorectal carcinomas compared to the adjacent tissue and normal tissue from the same individual ($p=3\times10^{-8}$). Also, the NDRG2 expression level in adjacent carcinoma tissue was lower than that of normal tissue. However, the expression pattern of c-MYC was the inverse ($p=5\times10^{-8}$). Finally, we induced the differentiation of the colorectal carcinoma cell lines HT29, SW480 and SW620 and found that NDRG2 expression increased and that c-MYC expression declined with increasing differentiation. These novel data show a disparity in both the mRNA and protein expression levels of Ndrg2 and c-Myc between colorectal cancers and normal tissues. Taken together, NDRG2 may play a role during the differentiation of colorectal cancer cells, and the function of NDRG2 in the development of colorectal cancer should be further investigated.

Key words N-Myc downstream-regulated gene 2; colorectal cancer; differentiation; Myc

Colorectal cancer (CRC) is a tumor type that accounts for 1020000 new cases and 530000 deaths worldwide per year. In the United States alone, there were 145000 new cases diagnosed per year, leading to 56000 deaths every year.[1,2] There were 146000 new cases of CRC in China, causing 78700 deaths, ranking CRC fifth out of all cancer-related deaths in China.[3] Approximately 70% of CRC tumors arise in the colon, whereas 30% occur in the rectum. Over the past several years, therapeutic options for patients with colorectal cancer have increased substantially, including earlier diagnosis, improved radical surgery, neoadjuvant chemotherapy, and anti-angiogenesis approaches.[4] However, despite these improvements, many colorectal cancers remain incurable. Efforts to better understand the biological basis for colorectal cancer progression may explain the wide variability observed between patients and thus may provide important, clinically relevant insights into disease management.

Mouse ndrg1 was first cloned from the embryos of N-myc knockout mice.[5] There are currently four members in the human NDRG family (N-Myc downstream-regulated gene): NDRG1, NDRG2, NDRG3, and NDRG4. We initially cloned human NDRG2 from a normal human brain cDNA library and found that NDRG2 is located on chromosome 14q11.2. Previous studies from our laboratory and others have shown that NDRG2 is more abundantly expressed in normal tissues than in tumors and cancer cell lines.[6—8] These data suggest that NDRG2 might be a novel tumor-related gene, the inactivation of which may play an important role in the initiation or progression of tumors.

In support of this idea, Lorentzen *et al.* recently reported that NDRG2 mRNA levels were lower in colorectal carcinomas and high-risk adenomas compared with normal tissue.[9] Although their work confirmed the decreased expression of NDRG2 mRNA in colorectal cancers, the change in mRNA level was not completely reflected at the protein level. Their research also did not evaluate c-MYC expression, which is closely associated with NDRG2 expression, or analyze the expression of NDRG2 in colorectal carcinomas at different stages of differentiation. Therefore, an analysis of NDRG2 and c-MYC mRNA and protein levels in colorectal carcinomas at different stages of differentiation would be necessary to determine their possible role in tumor development.

The oncogene MYC was first identified in 1978 as a cellular homolog of the viral oncogene myc from the retrovirus MC29 by Bishop *et al.*[10] Several research groups subsequently determined that the cellular myc (c-MYC) proto-oncogene is activated in various animal and human tumors and is amplified in about 70% of colorectal cancers.[11] Uncontrolled amplification of MYC causes abnormal proliferation that deregulates the cell cycle. Knock-out of c-myc or N-myc in mice results in embryonic lethality, suggesting that these genes are critical for development.[12,13] Furthermore, the overexpression of Myc induces cell proliferation and inhibits cell differentiation. Our previous work showed that Myc repressed human NDRG2 *via* a Miz-1-dependent interaction with the core promoter of NDRG2.[14] Given the fact that Myc is a pivotal regulatory master switch for cell proliferation and differentiation, we were eager to investigate the

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expression of Myc and NDRG2 in colorectal cancers. We previously reported an inverse regulatory relationship between human NDRG2 and MYC gene expression events during the induced colon cell differentiation.[14] It is, therefore, of great interest to confirm the relationship of Myc and NDRG2 expression in human CRC samples.

In the present study, we determined the expression pattern of NDRG2 and c-MYC in colorectal carcinoma specimens, corresponding adjacent tissues, and normal tissues. In addition, we investigated the expression of NDRG2 and c-MYC after inducing differentiation in colorectal cancer cell lines. These studies provide explicit evidence of the regulatory relationship between NDRG2 and c-MYC in colorectal cancer cells and indicate that NDRG2 may have an important role during the differentiation of colorectal cells.

MATERIALS AND METHODS

Tissue Collection The study was approved by the ethics committee of the Fourth Military Medical University. Based on the histology of colorectal cancer, we divided all specimens into three groups: poorly differentiated, moderately differentiated, and well-differentiated. Assuming that the test power was 0.75 and that the significance level was 0.05, we calculated the total sample size to be 210, with 70 cases in each group. Fresh colorectal carcinoma specimens, corresponding adjacent tissues, and normal tissues were obtained from 213 patients, including 32 poorly differentiated, 90 moderately differentiated, and 91 well-differentiated tumors. These patients all underwent surgery at the Department of Gastrointestinal Surgery of Xijing Hospital, the Fourth Military Medical University (Xi'an, China) between October 2005 and September 2007. The histomorphology of all specimens was confirmed by the Department of Pathology, Xijing Hospital. Patient clinical information, such as gender, age, family history, tumor location, differentiation, and TNM stage, was collected and stored in a database. Adjacent tissue was defined as that being within 2 cm of macroscopically unaffected margins of the tumor, and normal tissue was located at least 10 cm away from the margins of the tumor or as far away as possible.[15,16] All fresh tissues were obtained within 10 min after surgical removal. Parts of the specimens were placed into liquid N_2 for 10 min, then into a $-70\,^{\circ}C$ ultrafreezer for mRNA and protein isolation. The remaining tissues were fixed in 10% formaldehyde and embedded in paraffin for histological sectioning.

Two colorectal cancer tissue arrays were also used (Shanghai Outdo Biotech Co., Ltd.). One array contained tissues of 50 patients, including colon cancer tissue and matched normal tissue. In this array, there were 31 male and 19 female samples, or 22 well-, 17 moderately, and 11 poorly differentiated specimens. The other array contained tumor tissues from 100 CRC patients, including cancer tissues and matched normal tissue. There were in this array 58 male and 42 female, 48 well-, 35 moderately, and 17 poorly differentiated specimens. Cancer and matched normal tissues were located next to each other on the array, and the corresponding patient clinical information was available for statistical analysis.

Immunohistochemistry Assay The immunohistochemical assay involved the use of the avidin–biotin–peroxidase method on tissue specimens and tissue arrays. All sections were deparaffinized in xylene and dehydrated through a graduated alcohol series before endogenous peroxidase activity was blocked with 0.5% H_2O_2 in methanol for 10 min. Nonspecific binding was blocked by incubating sections with 10% normal goat serum in phosphate-buffered saline (PBS) for 1 h at room temperature. Without washing, sections were incubated with anti-Ndrg2 (1 : 100, Abnova) or anti-c-Myc (1 : 100, Santa Cruz) in PBS at 4 °C overnight in a moist box. Biotinylated goat anti-mouse IgG (1 : 400, Sigma) or goat anti-rabbit IgG (1 : 400, Sigma) was incubated with the sections for 1 h at room temperature and detected with a streptavidin–peroxidase complex. The brown color indicative of peroxidase activity was developed by incubating with 0.1% 3,3-diaminobenzidine (Sigma) in PBS with 0.05% H_2O_2 for 5 min at room temperature. The tissue specimens and two experimental arrays were evaluated separately by two pathologists using a double blind method. Five view fields in each sample were scored, and the average was considered in the statistical analysis. For scoring, a 0—3 score system was applied: A score of 0 indicates that less than 10% of the cells stained as positive. Scores of 1—3 indicate the intensity of the staining in cases where more than 10% of the cells were positive; a score of 1 corresponds to weak staining, 2 is moderate staining, and 3 is strong staining.[17]

Cell Culture and Differentiation Induction The human colon carcinoma cell lines HT29, SW480, and SW620 were obtained from the American Type Culture Collection. HT29 cells were cultured in 1640 medium (Sigma-Aldrich), while SW480 and SW620 cells were cultured in L-15 medium (Sigma-Aldrich). The media were supplemented with 10% fetal bovine serum (Atlanta Biologicals), and cells were cultured at 37 °C in 5% CO_2. Differentiation was induced by treatment with 2 mM sodium butyrate (Sigma Chemical) for 6 d, and cells were harvested each day from the beginning of treatment to the sixth day. The differentiation stage was assessed by transmission electron microscopy (for changes in cell structure and architecture) and alkaline phosphatase (AP) activity, as markers of differentiation. After removal of the culture medium, the attached cells were scraped into ice-cold PBS, centrifuged at $4000\times\boldsymbol{g}$, resuspended in PBS, and sonicated with an ultrasonic cell disrupter. Cellular debris was pelleted by centrifugation, and supernatants were transferred to new tubes and stored at $-70\,^{\circ}C$ until measurement. AP activity was measured according to the manufacturer's instructions using *p*-nitrophenylphosphate as substrate (Merck, Darmstadt, Germany) and calculated in units per milligram protein (U/mg prot). Protein content was determined using a BCA™ Protein Assay Kit.

Real-Time Polymerase Chain Reaction (PCR) SYBR green I technology was used for the real-time PCR analysis. Total RNA was purified from tissues and cells as recommended by the manufacturer using Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.). cDNA synthesis was performed using approximately 5 μg RNA per 20 μl using a cDNA reverse transcription kit (Fermentas). Real-time PCR was performed on an ABI 7500 system (Applied Biosystems). NDRG2 and c-MYC primers were 5′-GAGATATGCTCTTAACCACCC-3′ and 5′-GCTGCCCAATCCATCCAA-3′, and 5′-GGAGGAACAAGAAGATGAGGAAG-3′ and 5′-

AGGACCAGTGGGCTGTGAGG-3′, respectively. The internal control β-actin primers were 5′-ATCATGTTTGAGACCTTCAACA-3′ and 5′-CATCTCTT-GCTCGAAGTCCA-3′. Primers were designed using Primer Express v3.0 Software. After first strand synthesis, an equivalent of 50 ng of starting total cellular RNA (1/10 of the cDNA reaction) was added to two duplicate PCR reactions containing 12.5 μl SybrGreen mix, 0.5 μl SybrGreen rox, 100 nmol/l forward primer, and 100 nmol/l reverse primer in a final volume of 25 μl. Each sample was used in a single reaction that cycled at 95 °C for 10 min (to activate enzyme), followed by 45 cycles of 95 °C for 10 s and 60 °C for 34 s on an ABI SDS 7500 system (Applied Biosystems). Fluorescent data were converted into cycle threshold measurements using the SDS system software. The $2^{-\Delta\Delta Ct}$ method was used as a measure for the relative expression of the target gene. NDRG2 and C-MYC mRNA levels were compared to actin.[18] Thermal dissociation plots were examined for biphasic melting curves, indicative of whether primer-dimers or other nonspecific products could be contributing to the amplification signal.

Western Blot Analysis Total tissue proteins were extracted in lysis buffer and then centrifuged at 12000×***g*** for 5 min at 4 °C. The supernatants were collected, and protein concentrations were determined, using Bio-Rad protein assay dye reagent (Bio-Rad). Aliquots (50 μg) of whole protein lysates were loaded onto sodium dodecyl sulfate polyacrylamide (10%) gels for electrophoresis. For Western blot analysis, proteins were transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences). The membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 and incubated with each primary antibody (Ndrg2, c-Myc, or actin) overnight at 4 °C. Finally, blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Promega) for at least 1 h at room temperature and detected using the ECL method (Amersham Biosciences).

Statistical Analysis The rank sum test was used to analyze the ranked data using the statistical package SPSS (version 11.0). The measurement data were analyzed by using one-way ANOVA. Randomized complete block design ANOVA was used to analyze the statistical difference among different tissue types. Spearman's analysis was carried out to determine the correlation between two markers. $p<0.05$ was considered statistically significant.

RESULTS

Ndrg2 Protein Levels Are Reduced in Colorectal Cancers Our previous data demonstrated that Ndrg2 levels are reduced in many types of cancer.[19] In the present study, we investigated this phenomenon in colorectal cancer. Ndrg2 expression was assessed in colorectal tissue, making use of a tissue array. Based on immunohistochemistry analysis and the hierarchical scores of the staining, we found a significant decrease in Ndrg2 expression in colorectal cancers as compared with normal colorectal tissues. In addition, Ndrg2 expression was found to be associated with the differentiation level of the tumors ($p=0.005$, Table 1), being low in normal and well-differentiated tissues and high in poorly differentiated colorectal carcinoma.

Ndrg2 expression was not significantly associated with gender, age, depth of tumor infiltration, lymph node aversion, or TNM stage of the tumors.

Table 1. Statistical Results of Tissue Array Immunohistochemistry

	n	Ndrg2				*p*[a]
		0	1	2	3	
	150	78	43	23	6	
Gender						
Male	89	42	24	10	4	0.801
Female	61	36	19	13	2	
Age						
<60	48	25	16	7	2	0.866
≥60	102	53	27	16	4	
Tumor location						
Right	19	10	5	3	1	0.802
Transverse	16	8	4	3	1	
Left	20	8	7	4	1	
Sigmoid	17	9	6	2	0	
Rectum	78	43	21	11	3	
Histology						
Poorly-differentiated	28	21	5	2	0	0.005
Moderately-differentiated	52	29	14	8	1	
Well differentiated	70	28	24	13	5	
Influence depth						
In muscular	37	21	11	4	1	0.378
Out of ectoptygma	113	57	32	19	5	
Lymph node aversion						
Negative	75	40	20	13	2	0.810
Masculine	75	38	23	10	4	
UICC stage						
I	21	11	6	3	1	0.543
II	54	25	18	9	2	
III	24	16	4	4	0	
IV	51	26	15	7	3	

a) *p* value when using Kruskal–Wallis test.

Ndrg2 and c-Myc Protein Levels Are Affected by Differentiation Status in Colorectal Carcinomas Encouraged by the observed decrease in Ndrg2 protein level in the colorectal cancer array, we collected 213 colorectal cancer samples together with patient-matched tissues adjacent to the tumor and normal colorectal tissues to analyze both the expression of Ndrg2 and c-Myc by immunostaining. In agreement with the tissue array, we detected higher expression of Ndrg2 in normal colorectal tissues (Fig. 1). The spatial distribution of Ndrg2 and c-Myc was mainly confined to the glandular epithelium cells, and it did not extend to the mesenchymal cells. Ndrg2 was localized in the cytoplasm and nucleus, which was in accordance with our previous findings.[20] Ndrg2 expression also decreased between well-differentiated tumors and poorly differentiated tumors. Myc expression followed the opposite pattern, being abundant in poorly differentiated colorectal carcinoma compared with the well-differentiated tumors and normal colorectal tissue. c-Myc was localized in the nucleus of colorectal tumors (Fig. 1), partly representing the activation mode of c-Myc.

Quantitative Analysis of Ndrg2 and c-Myc Protein Expression Based on Differentiation Status in Colorectal Carcinomas To quantitatively analyze differences in Ndrg2 and c-Myc expression in colorectal tumors, we performed Western blot analysis on tumor and normal tissue. We detected an increasing trend of Ndrg2 in 136 colorectal cancers, while a decreasing trend of c-Myc was detected in 151 cases. As shown in Fig. 2A, we found that Ndrg2 protein

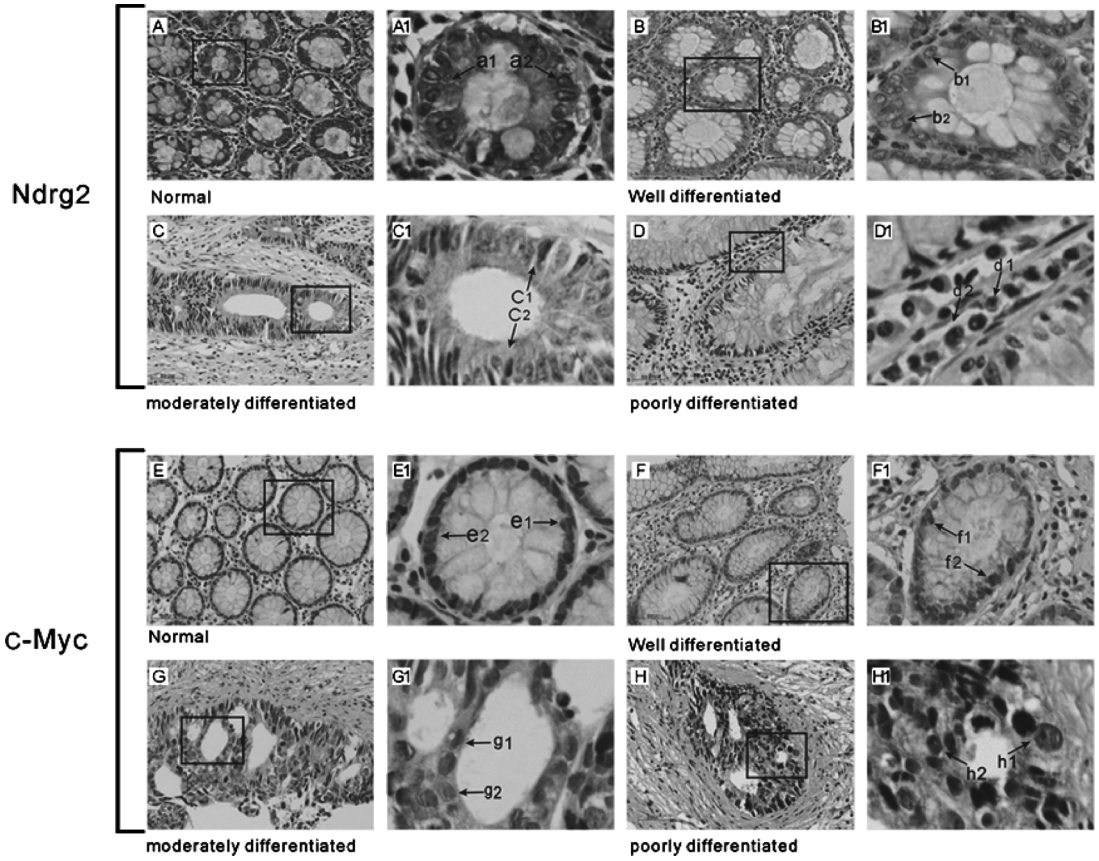

Fig. 1. Expression of Ndrg2 (A—D) and c-Myc (E—H) in Colorectal Cancer Tissues by Immunohistochemistry

Ndrg2 staining was found mainly in the cytoplasm (a1, a2, b1, b2, c1, c2, d1, d2), while c-Myc staining was mainly in the cell nucleus (e1, e2, f1, f2, g1, g2, h1, h2). Ndrg2 staining was strong in normal colon tissue (A) and decreased gradually from well-differentiated (B), *via* moderately differentiated (C), to poorly differentiated (D) colorectal cancer tissues. c-Myc staining was weak in normal colon tissue (E) and increased gradually from well-differentiated (F) to moderately differentiated (G) to poorly differentiated (H) colon cancer tissues. Pictures were amplified to ×400, each scale represents 50 μm.

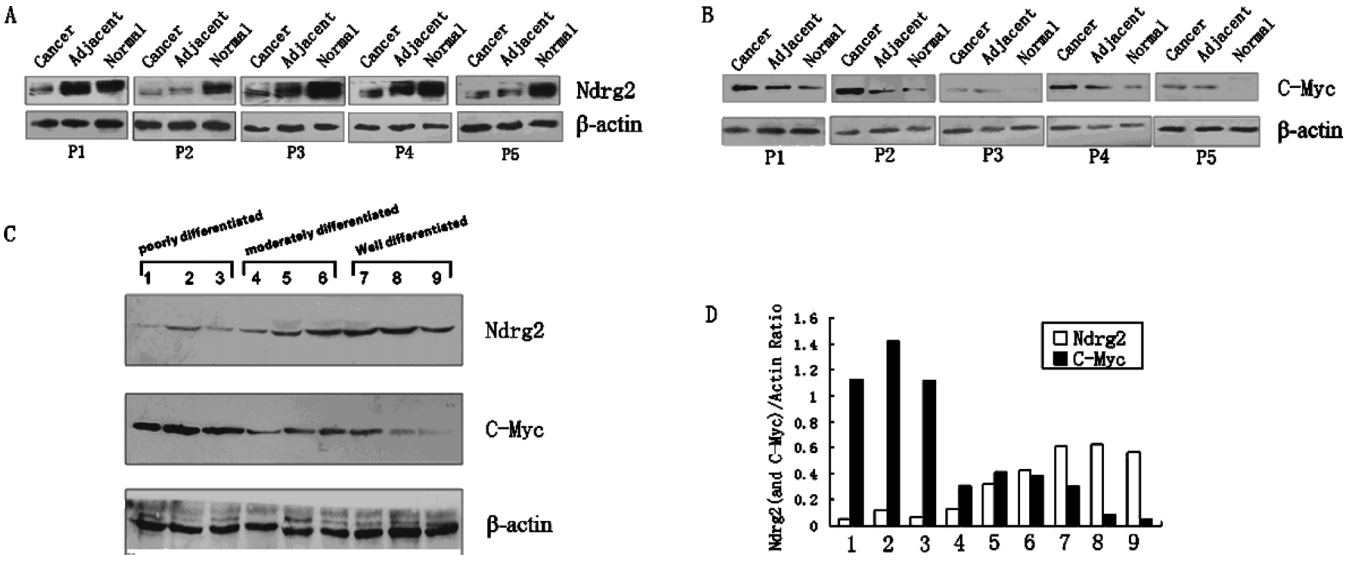

Fig. 2. Expression of Ndrg2 and c-Myc Protein in Cancerous Tissues, Adjacent Tissues and Normal Tissues of the Same Patients by Western Blot Analysis

(A) Ndrg2 expression level in cancerous, adjacent, and normal tissues of 5 patients. (B) c-Myc expression level in specimens from the same patient as A. (C) Ndrg2 and c-Myc expression in 3 poorly, 3 moderately, and 3 well-differentiated cancer tissues, respectively, which was indicated by the ratio of Ndrg2/Actin and C-Myc/Actin (D). β-Actin was used as a control for equal protein loading.

in the tumors was expressed at lower levels as compared to the normal colorectal tissues of the same individuals. This result confirmed the immunohistochemical assessment of Ndrg2 expression. Furthermore, we observed an increased expression of c-Myc in the normal tissues (Fig. 2B). Combining the findings of Ndrg2 and Myc protein in the colorectal tumors compared with normal tissue, we conclude that Ndrg2 expression is suppressed in colorectal cancer.

Next, we investigated whether the expression of Ndrg2 correlated with the differentiation level. Ndrg2 expression increased, and c-Myc expression steadily decreased, from poorly, through moderately, to well-differentiated grades (Fig. 2C). This result is in agreement with the findings of the immunohistochemistry analysis and indicates a close correlation of Ndrg2 protein expression with differentiation. These results suggest that Ndrg2 is involved in the differentiation process of colon epithelial cells.

We subsequently determined the mRNA expression of NDRG2 and c-MYC, normalized to β-actin, by quantitative real-time RT-PCR. As shown in Table 2, we observed a decrease in the expression of NDRG2 mRNA in the colorectal tissues, as compared to the normal colorectal tissue and the adjacent tissue ($p=3\times10^{-8}$). As we have already recognized that Myc can transcriptionally repress the activity of NDRG2,[14] we also examined the expression of c-MYC mRNA. We found the highest expression of c-MYC in colorectal cancers, which was the inverse of the result for NDRG2 expression. NDRG2 mRNA expression increased from poorly, through moderately, to well-differentiated tumors ($p=2\times10^{-6}$). In contrast, c-MYC mRNA expression

Table 2. Mean Value of Normalized NDRG2 and c-MYC mRNA Level in Specimens with Different Tissue Type and Differentiation

	n	NDRG2 Mean±S.D.	p	c-MYC Mean±S.D.	p
Tissue type					
Carcinoma	213	1.069±0.214	$3\times10^{-8\,a)}$	5.688±0.356	$5\times10^{-8\,a)}$
Adjacent	213	3.346±0.512		3.397±0.519	
Normal	213	5.645±0.344		1.114±0.210	
Histology					
Poorly differentiated	32	0.825±0.104	$2\times10^{-6\,b)}$	6.148±0.114	$3\times10^{-7\,b)}$
Moderately differentiated	90	1.055±0.099		5.819±0.105	
Well differentiated	91	1.168±0.113		5.396±0.112	

a) *p* value when using randomized complete block design ANOVA. *b*) *p* value when using one-way ANOVA.

Table 3. Mean Value of Normalized NDRG2 and c-MYC mRNA Level in Specimens with Different Gender, Age, Infiltrate Depth, Lymph Node Aversion and Dukes Stage

	n	NDRG2 Mean±S.D.	$p^{a)}$	c-MYC Mean±S.D.	$p^{a)}$
Gender					
Male	125	1.055±0.189	0.211	5.693±0.319	0.785
Female	88	1.089±0.203		5.681±0.312	
Age					
<60	83	1.062±0.197	0.707	5.711±0.305	0.430
≥60	130	1.073±0.215		5.673±0.364	
Family history					
Positive	8	1.072±0.211	0.918	5.732±0.308	0.378
Negative	205	1.069±0.209		5.681±0.371	
Tumor location					
Right	25	1.066±0.198	0.734	5.672±0.368	0.572
Transverse	19	1.056±0.192		5.683±0.373	
Left	27	1.061±0.195		5.702±0.375	
Sigmoid	24	1.064±0.197		5.741±0.376	
Rectum	118	1.073±0.207		5.679±0.369	
Infiltrate depth					
In muscular	57	1.085±0.217	0.479	5.697±0.324	0.821
Out of ectoptygma	156	1.063±0.194		5.685±0.349	
Lymph node aversion					
Negative	122	1.072±0.223	0.820	5.691±0.313	0.871
Masculine	91	1.065±0.220		5.684±0.305	
UICC stage					
I	30	1.073±0.216	0.953	5.712±0.321	0.837
II	91	1.086±0.184		5.673±0.311	
III	50	1.066±0.197		5.702±0.325	
IV	42	1.071±0.208		5.688±0.316	

a) *p* value when using one-way ANOVA.

decreased (Table 2). There was a significant negative correlation between the expression of NDRG2 and c-MYC mRNA ($r_s=-0.675$, $p=1\times10^{-5}$). No correlations were found with other patient-related factors, such as gender, age, depth of infiltration, lymph node aversion, and TNM stage of the tumors (Table 3).

NDRG2 Expression Increases in Colon Carcinoma Cells after Inducing Differentiation To investigate the expression of Ndrg2 during colon cell differentiation, *in vitro* studies with HT-29, SW620, and SW480 colon cancer cell lines were performed. Cells were treated with 2 mM sodium butyrate to induce differentiation.[21] Following the incubation, the differentiation status was confirmed by examining the presence of regular brush borders and tight junctions, which are structural markers of differentiation (Fig. 3A), using transmission electron microscopy. In addition, butyrate-induced differentiation was measured by alkaline phosphatase (AP) activity. AP activity increased significantly in SW620 and SW480 cells after 48 h of incubation with butyrate and after 96 h in HT-29 cells (Fig. 3B). Both protein and mRNA expression of NDRG2 and c-MYC were measured in these cell lines at the indicated time points after butyrate treatment. Prolonged incubation with butyrate resulted in a time-dependent induction of Ndrg2 expression (Fig. 4A). Conversely, increasing differentiation induced a decrease in c-Myc expression in a time-dependent pattern (Fig. 4B). Real-time PCR analysis confirmed these results at the mRNA level (Figs. 4C, D).

DISCUSSION

Our previous data and reports by others have described the complete or partial loss of NDRG2 expression in tumors and tumor cell lines. Integrative genomic analysis identified the inactivated NDRG2 in aggressive meningioma. Ndrg2 showed 21% and 77% loss in biologically benign and aggressive meningiomas, respectively.[10] Also, Ndrg2 was not expressed in 151 of 286 gastric cancers.[22] We have previously confirmed these observations of suppressed Ndrg2 in human glioblastomas and glioblastoma cell lines.[23] Here, we show at the RNA and protein levels that NDRG2 expression is reduced in human colorectal cancer, while, at the same time, c-Myc is increased. Our results strongly agree with Lorentzen *et al.*,[9] who indicated the importance of Ndrg2 in colorectal

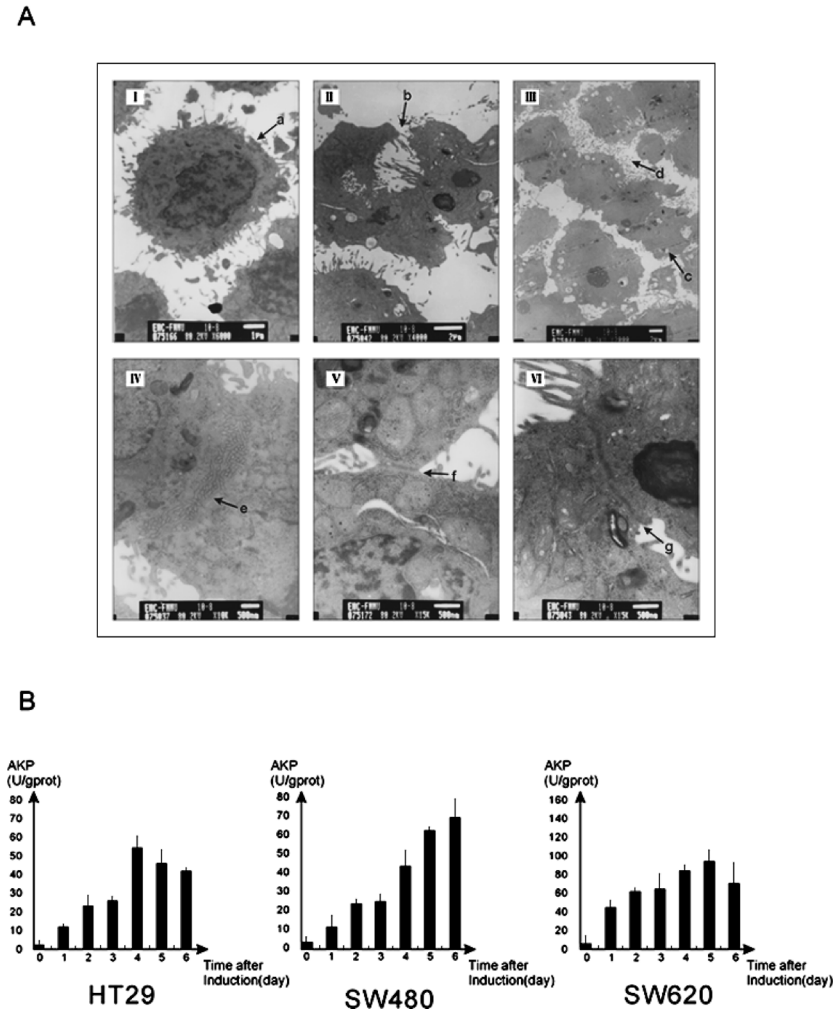

Fig. 3. (A) Electron Microscopy of Colon Cancer Cells during the Process of Induced Differentiation

I undifferentiated colon cells; II—VI changes in ultramicrostructure during colon cell differentiation. (a) Undifferentiated cell. (b) Cryptae structure. (c) Disciplinary conjunction between cell. (d) Polarisation of microvilli. (e) Cryptae structure between cells. (f) Macula adherens in cell. (g) Tight junction and macula adherens between cells.

(B) AP Level during Induced Cell Differentiation in Three Different Colon Cancer Cell Lines

AP levels in cell lines HT29, SW480, and SW620 were measured from the 1st to the 6th day during the induced differentiation process compared with untreated cells (day 0).

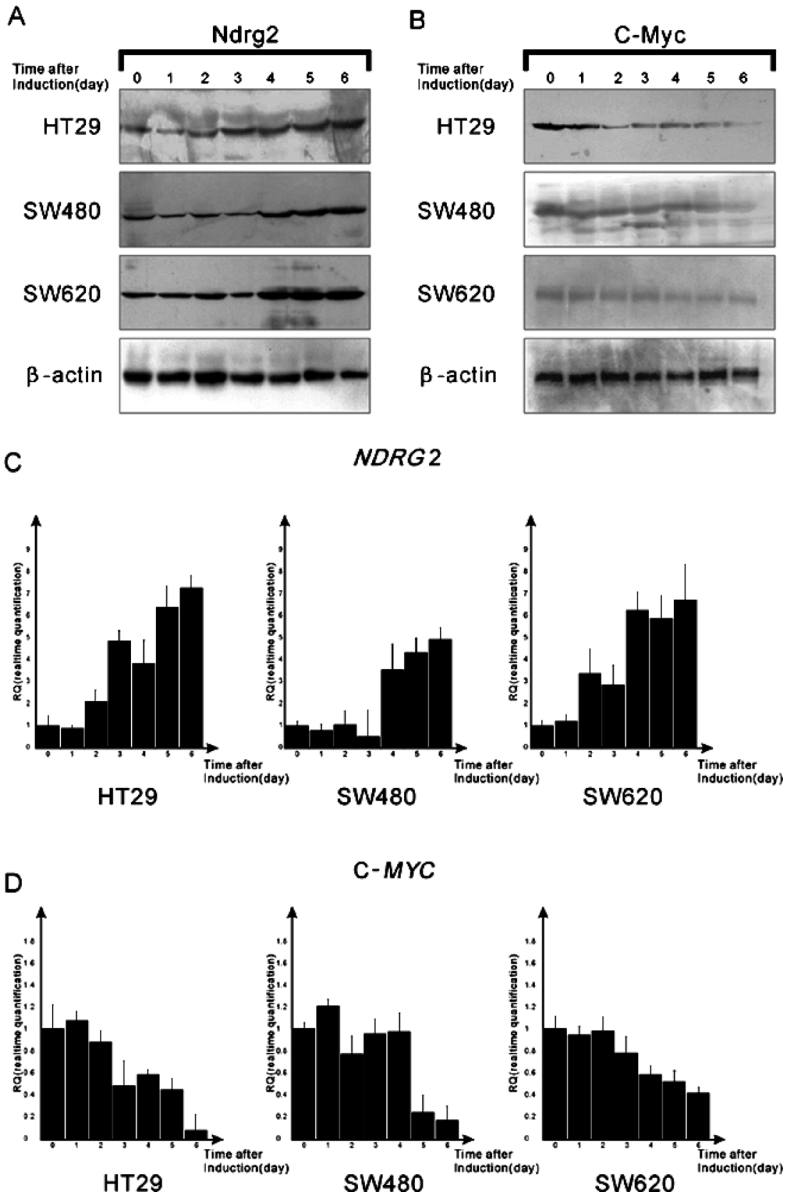

Fig. 4. (A, B) Ndrg2 and c-Myc Protein Level by Western Blot Analysis during Induced Cell Differentiation

The differentiation of HT-29, SW480, and SW620 colon cancer cells was induced as described in Materials and Methods. (A) Ndrg2 protein level each day from the 1st to the 6th day compared with untreated cells (day 0). (B) c-Myc protein level at the same time points as Ndrg2. β-actin was used as a control for equal protein loading.

(C, D) Expression of NDRG2 and c-MYC mRNA during Induced Cell Differentiation by Real-Time PCR

(C) *NDRG2* mRNA level by real-time PCR from the 1st to 6th day of induced differentiation compared with untreated cells (day 0), normalized to β-actin. (D) c-*MYC* mRNA level by real-time PCR at the same time points as *NDRG2* compared with untreated cells (day 0), normalized to β-actin.

cancer progression.

Myc, as a master switch molecule for cell proliferation and differentiation, exerts its biological function mainly by transcriptional regulation of its target genes.[24,25] Indeed, we have shown that Myc is able to repress human NDRG2 at the transcriptional level. As the reverse regulatory target of Myc, the function of NDRG2 might inhibit cell proliferation and promote differentiation.[26] Therefore, profiling the expression of NDRG2 as well as Myc in colorectal cancers and exploring their regulatory relationship in the differentiation of colorectal cells may provide important insights. Our current results regarding NDRG2 and Myc, obtained from 213 cases of human colorectal cancers, including poorly differentiated ($n=32$), moderately differentiated ($n=90$) and well-differentiated carcinomas ($n=91$), are in agreement with previous reports. While NDRG2 expression was found to be reduced in colorectal cancers, as compared with adjacent and normal colorectal tissue from the same individuals ($p=3\times10^{-8}$), a reversed expression pattern was observed for c-Myc, being abundant in carcinoma tissues and suppressed in normal tissues. These results suggest that the transcriptional repression of human NDRG2 by c-Myc might participate in the carcinogenesis of the colorectal cancer.[26] As a downregulated target of oncogenic Myc, we believe that NDRG2 is involved in inhibition of tumor cell proliferation. NDRG2 may, therefore, be a potential target for gene therapy of (colorectal) cancer.

Other reports have suggested that Ndrg2 could promote cell differentiation. During treatment with maturation-induc-

ing stimuli, Ndrg2 expression increased during the differentiation of monocyte-derived dendritic cells.[27] In PC12 cells, Ndrg2 overexpression caused neurite elongation after treatment with nerve growth factor.[28] We also observed induction of NDRG2 expression upon differentiation of colorectal tumors. NDRG2 mRNA and protein levels increased gradually in time and with differentiation status. The changes in NDRG2 expression during the differentiation of colon cancer cells may provide clues regarding its function.

Taken together, our results revealed that NDRG2 and c-MYC are expressed differently in normal tissues, adjacent tissues, and cancer tissues from the same patient and that NDRG2 expression is the highest in well-differentiated colorectal carcinomas. These data suggest that NDRG2 may have an important role in the development of colorectal cancer. Although the present data do not identify NDRG2 as an explicit predictive or prognostic biomarker, they do provide __ evidence that NDRG2 expression correlates with the differentiation level of CRC, which suggests an important role in tumor biology.

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