

NDRG2 positively regulates E-cadherin expression and prolongs overall survival in colon cancer patients

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Abstract. To discover the molecular mechanism of N-Myc downstream-regulated gene 2 (NDRG2), a newly found differentiation-related tumor suppressor, the relationships between NDRG2 and E-cadherin were investigated in tumor cells and tissues. Positive correlations between the expression of E-cadherin and NDRG2 were shown in several colon cancer cell lines as well as in colon cancer tissues. According to the transcription assays using a reporter plasmid containing *E-cadherin* promoter region (-368~+51), NDRG2 introduction into colon cancer cell lines induced upregulation of *E-cadherin* promoter activity and its transcription. On the contrary, inhibition of NDRG2 expression by siRNA treatment caused the decrease of *E-cadherin* transcription. Snail, a zinc-finger transcriptional repressor, was shown to be a mediator of NDRG2-regulated E-cadherin expression. The enhancement of glycogen synthase kinase 3 β (GSK-3 β) activity by NDRG2 overexpression caused proteasomal degradation of Snail

transcription factor followed by transcriptional de-repression of *E-cadherin*. We also found that NDRG2 could mediate cell density-regulated E-cadherin expression. The increase of NDRG2 expression with cell density preceded E-cadherin expression, and the regulation of Snail activity by GSK-3 β was also related to this process.

Introduction

N-Myc downstream-regulated gene 2 (NDRG2) is generally expressed in the brain, heart and muscle (1). Increasing evidence indicates that NDRG2 has specific functions in the regulation of cellular differentiation and tumorigenesis. Indeed, NDRG2 was reported to have a putative role in neural differentiation, synapse formation, and axon survival in response to glucocorticoids, and to participate in the differentiation of monocytes and leukemia cells into dendritic cells (2,3). NDRG2 was discovered as a potential tumor suppressor in meningioma, glioblastoma and melanoma cell lines (4-6). In addition, NDRG2 mRNA and protein are shown to be downregulated in human liver, pancreatic and colon cancer tissues (7,8). Recently, NDRG2 was identified as a potential suppressor of tumor metastasis in highly malignant tumor cells (9,10). NDRG2 inhibited the invasion and migration of highly invasive tumor cells both *in vitro* and *in vivo*.

Aggressive cancer progression is initiated by the breakdown of epithelial cell homeostasis which is correlated with the loss of epithelial characteristics and the acquisition of a migratory phenotype such as mesenchymal cells (11). This phenomenon, known as epithelial-mesenchymal transition (EMT), is considered to be a critical event during tumor cell malignancy (11-14). Epithelial cells are connected to each other through specialized structures known as adherens junctions, which are composed of E-cadherin/ α - and β -catenin complexes. The development of metastatic carcinoma is accompanied by deregulation of adherens junctions and the decrease of E-cadherin expression is an early step during the initiation of these processes.

Loss of *E-cadherin* gene expression or E-cadherin protein is frequently found during tumor progression in most types of epithelial cancer. Mutation or inactivation (by DNA methylation) of *E-cadherin* gene is responsible for such changes. However, in several types of cancer, E-cadherin expression is lost without genomic modifications (15) owing to transcrip-

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Abbreviations: NDRG2, N-Myc downstream-regulated gene 2; EMT, epithelial-mesenchymal transition; GSK-3 β , glycogen synthase kinase 3 β ; TCF, T-cell factor; HMGA2, high mobility group A2; CD, cluster designation; PVDF, polyvinylidene fluoride; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IgG, immunoglobulin G; mAb, monoclonal antibody; RT-PCR, reverse transcriptase-polymerase chain reaction; RNA, ribonucleic acid; dNTP, deoxyribonucleotide triphosphate

Key words: N-Myc downstream-regulated gene 2, E-cadherin, epithelial-mesenchymal transition, glycogen synthase kinase 3 β phosphorylation, snail

tional repression of *E-cadherin* by those EMT-regulating factors (16-20). An increasing number of transcription factors appear to activate EMT in various settings, including Snail (16-18,20,21), Twist (22), high mobility group A2 (HMGA2) (23), Slug (24,25), SIP1 (19) and Ets-1 (26). A central role of these transcriptional regulators is the suppression of the *E-cadherin* gene. Downregulation of E-cadherin has several important consequences that are of direct relevance to EMT.

Several signaling pathways implicated in the progression of EMT, including the Wnt and phosphoinositide 3-kinase pathways, use glycogen synthase kinase-3 β (GSK-3 β) to mediate their responses (27,28). We previously discovered that NDRG2 expression correlates with tumor stages, and NDRG2 can control tumor development by regulating GSK-3 β activity to modulate TCF/ β -catenin signaling (8). According to recent studies, the expression of Snail family transcription factors and the following EMT process were shown to be regulated by the Wnt/ β -catenin pathway (29-31). In the present study, we found the functional relevance of NDRG2 on GSK-3 β activity and the following EMT processes.

Materials and methods

Cell lines and transfection. All cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA); KM12c, Colo205, HCT116, HT29, SW480 and SW620. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL, Invitrogen, Carlsbad, CA, USA) supplemented with 2 mM glutamine, 1% penicillin/streptomycin and 10% fetal bovine serum (FBS; HyClon, Logan, UT, USA), and kept at 37°C in a humidified incubator which was maintained with 5% CO₂. Plasmid containing wild-type NDRG2 coding region (32) was transfected into the cell lines with Lipofectamine plus reagent (Invitrogen) according to the manufacturer's instructions. Small interfering RNA directed against human NDRG2 (siGENOME SMARTpool, catalog No. M-013858-00) was purchased from Dharmacon (Thermo Fisher Scientific Inc., Waltham, MA, USA) with its control non-targeting siRNA (catalog No. D-001210-01), and treated according to the manufacturer's instructions. As an example, 12 pmole of NDRG2 siRNA was mixed with 15 μ l of Lipofectamine RNAiMAX reagent (Invitrogen), following the instructions, incubated for 20 min at RT, and treated onto cell lines plated on a 60-mm dish with 50% confluence.

Antibodies and western blotting. Cells were washed with phosphate-buffered saline (PBS), and lysed with cell lysis buffer [20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% NP-40, 2.5 mM sodium pyrophosphate, 1 mM Na₃VO₄, 1 mM NaF and Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN, USA)] on ice for 30 min. Thirty to fifty micrograms of the lysate were resolved by SDS-PAGE on 10 or 12% gels and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were incubated with primary antibodies followed by peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin (IgG) antibodies (Calbiochem, EMD Chemicals Inc., San Diego, CA, USA) and SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Thermo Fisher

Scientific, Rockford, IL, USA) for band visualization. To verify equal loading and adequate transfer, the membranes were probed with anti- α -tubulin or anti-GAPDH antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The primary antibodies were anti-E-cadherin (BD Biosciences, San Jose, CA, USA), anti-GSK-3 β , anti-phospho-GSK-3 β (Cell Signaling Technology, Beverly, MA, USA) and anti-Snail (Santa Cruz Biotechnology). Monoclonal anti-NDRG2 antibody (clone# 18c12) was previously described (33).

Luciferase reporter assay. Cells at 40-50% confluence in 12-well plates were transfected with a reporter luciferase plasmid containing *E-cadherin* promoter region (GeneBank ID: L34545, -368~+51) (34) in combination with NDRG2-expressing plasmid using FuGENE 6 transfection reagent (Roche) according to the manufacturer's instructions. Total amount of plasmid DNA per well was adjusted to be the same by adding suitable amounts of empty vector. Cells were harvested 48 h after transfection, and luciferase activity was measured by commercial luciferase assay kit (Promega, Madison, WI, USA) on TD-20/20 Turner luminometer (Turner Designs, Sunnyvale, CA, USA). Transfection efficiency was normalized by co-transfection of β -galactosidase with minimal promoter (pSV- β -galactosidase; Promega). All experimental and control groups contained at least three wells, and the results were reported as mean absorption \pm standard error.

RT-PCR analysis. Two-step RT-PCR reaction was performed using reverse transcriptase with oligo-dT primer and *Taq* polymerase (Takara Bio, Shiga, Japan) with specific primer pairs. Total RNA was isolated by a standard protocol (35), and cDNA was synthesized using AccuScript High Fidelity first strand cDNA synthesis kit (Stratagene, La Jolla, CA, USA) following the manufacturer's instructions. One microliter of the synthesized cDNA was used per 20 μ l PCR reaction, which comprised of 0.2U *ExTaq* DNA polymerase, 1X buffer and 1 mM dNTP mix (Takara) with specific primer pair, and amplified as follows: 94°C for 5 min, then 25-40 cycles of 94°C for 45 sec, 56°C for 45 sec and 72°C for 1 min, followed by a final extension of 7 min at 72°C using GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA). The PCR primers were designed by the Primer3 program and purchased from Bioneer Corp. (Daejeon, Korea). The PCR products were separated on 1.5% agarose gel, stained with ethidium bromide, visualized by Gel Doc 2000 UV transilluminator (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed using Quantity One software (Bio-Rad Laboratories). Each sample was tested more than three times and representative data are shown. The used primer pairs were: NDRG2, 5'-GGACATCTTTTCAGC-CAGGA-3' (F) and 5'-CCCATGCCTTGCAGGAAGT-3' (R); GAPDH, 5'-CCATCACCATCTTCCAGGAG-3' (F) and 5'-ACAGTCTTCTGGGTGGCAGT-3' (R); E-cadherin, 5'-TGAT TCTGCTGCTCTTGCTG-3' (F) and 5'-CGAGTCCCCTAGTC GTCCT-3' (R); Snai-1, 5'-CACTATGCCGCGCTCTTT-3' (F) and 5'-GGCAGGTATGGAGAGGAAGA-3' (R).

Patient samples and immunohistochemistry (IHC). Human colorectal carcinoma samples were obtained from patients who underwent routine surgery for colorectal cancer at the Department of Surgery, Eulji University Hospital, between

January 2002 and December 2005. For immunohistochemical study, 99 colorectal carcinoma tissues and paired normal mucosal tissues obtained from a site distant from the tumor lesion were fixed in 10% neutralization-buffered formalin solution for 24 h and embedded in paraffin wax. Serial sections 4- μ m thick were cut and mounted on charged glass slides (Superfrost Plus; Fisher Scientific, Rochester, NY, USA). IHC conditions for NDRG2 and E-cadherin were optimized and evaluated by two independent pathologists. In brief, tissue sections were microwaved twice for 10 min in citrate buffer (pH 6.0) for antigen retrieval. The sections were then treated with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity followed by incubation with 1% BSA. Mouse monoclonal antibody against NDRG2 and E-cadherin (Clone 36B5; Neomarkers, Fremont, CA, USA), was used at dilutions of 1:100 and 1:30, respectively. The avidin-biotin detection method was used and the tissue section was immersed in 3-amino-9-ethyl carbazole (AEC) as a substrate, and counterstained with 10% Mayer's hematoxylin, dehydrated and mounted by crystal mount. An irrelevant mouse IgG of the same isotype or antibody dilution solution served as a negative control.

Assessment of immunostaining and statistical analysis. Each slide was evaluated for NDRG2 and E-cadherin immunoreactivity using a semi-quantitative scoring system for both the intensity of the stain and the percentage of positive neoplastic cells. NDRG2 and E-cadherin immunoreactivity was observed primarily in the cytosolic membrane in colorectal mucosal and malignant cells. The intensity of membrane staining was coded as: 0, lower than the adjacent normal-appearing mucosal epithelium; 1, similar to the adjacent mucosal epithelium; and 2, stronger than the adjacent mucosal epithelium. The percentage of cells displaying a stronger staining intensity than the adjacent mucosal epithelium was scored as: 1 (0-24% tumor cells stained), 2 (25-49% tumor cells stained), 3 (50-74% tumor cells stained), 4 (75-100% tumor cells stained). For the purpose of statistical analysis, the median of this series (25% of malignant cells showing a stronger intensity than adjacent colonic epithelium) was used as a cut-off value to distinguish tumors with a low (<25%) or high (>25%) level of NDRG2 and E-cadherin expression. The relationship between the results of the immunohistochemical study and the clinicopathological parameters was determined using the SPSS software package (version 14.0; SPSS Inc., Chicago, IL, USA). The correlation between staining index scores and other categorical factors was analyzed using the Pearson's Chi-square test of independence.

Prognostic parameter for overall survival. Recurrence free survival was defined as the time from the date of surgery to the first date of recurrence of cancer, or mortality due to any cause. Overall survival was defined as the time from the date of surgery to the date of last follow-up or mortality due to any cause. The median follow-up period for all patients was 53.3 months (inter-quartile range, 23.5-77.0). Survival curve and median survival curve were estimated by the Kaplan-Meier method. The log-rank test was used to evaluate the statistical significance of differences in survival distribution. Multivariate analysis was performed using the Cox propor-

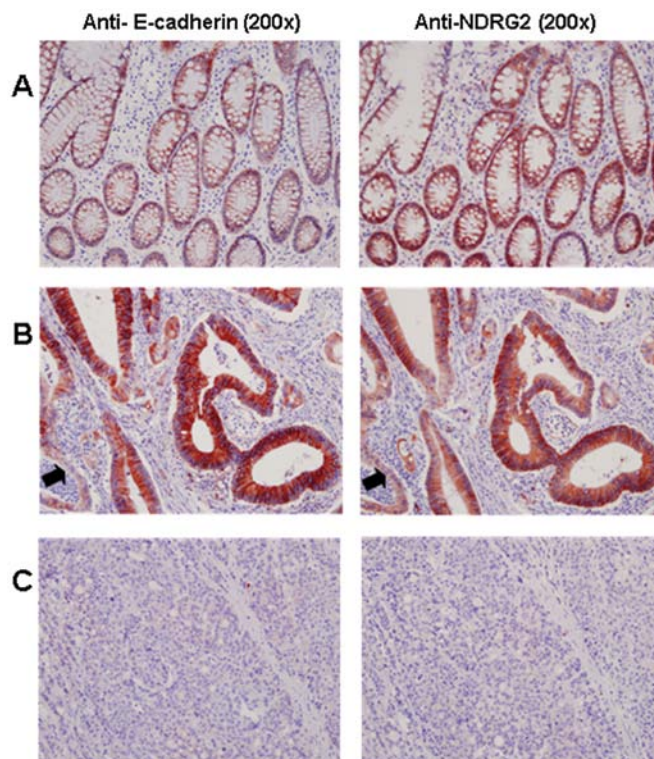


Figure 1. Expressional correlation between NDRG2 and E-cadherin in human colon carcinoma. Immunohistochemical staining with anti-NDRG2 antibody and anti-E-cadherin antibody. (A) NDRG2 and E-cadherin were highly expressed in the normal colonic mucosa. (B) NDRG2 and E-cadherin were strongly expressed primarily on the cytosolic membrane of tumor cells in well-differentiated adenocarcinoma areas, but weakly or negatively expressed in less-differentiated carcinoma areas (arrowhead). (C) In poorly differentiated adenocarcinoma, tumor cells showed negative staining with anti-NDRG2 and anti-E-cadherin, respectively.

tional hazards regression analysis. Results were considered to indicate a statistically significant difference if $P < 0.05$.

Confocal microscopy. Cells were cultured on coverslips, rinsed three times in cold PBS, fixed with 4% paraformaldehyde at room temperature for 20 min and permeabilized with 0.1% Triton X-100 in PBS for 10 min. The cells were then blocked with 1% BSA in PBS for 30 min, and stained with anti-E-cadherin and anti-NDRG2 mAbs for 2 h. Finally, the cells were incubated with Alexa Fluor 488 or 594 conjugated rabbit anti-mouse IgG (Molecular Probes, Invitrogen) in darkness for 1 h. The coverslips containing the cells were mounted on glass slides with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA) and visualized using Zeiss confocal microscope LSM 510 META (Carl Zeiss, Jena, Germany) at x40 magnification. The confocal images were captured by Zeiss LSM Image Browser program.

Results

The relationship between NDRG2 and E-cadherin expression. Expression levels and patterns of NDRG2 and E-cadherin in human colorectal carcinomas were evaluated by immunohistochemical analysis. Fig. 1A shows that both NDRG2 and E-cadherin were highly expressed in a similar pattern in normal colonic mucosa from patients in cytoplasmic membrane.

Table I. Correlation between NDRG2 and E-cadherin expression status.

	Frequency	Total	E-cadherin expression	
			Low/negative, n (%)	High, n (%)
Membranous NDRG2 expression	Low, n (%)	55	30 (54.5)	25 (45.5)
	High, n (%)	44	2 (4.5)	42 (95.5)

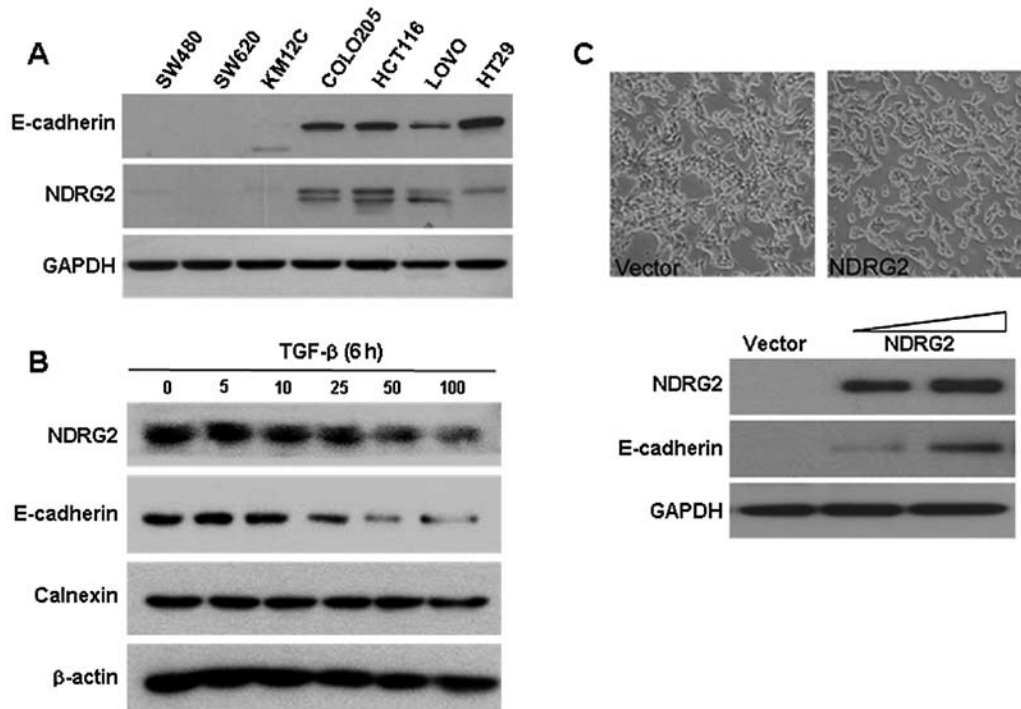


Figure 2. Analysis of E-cadherin and NDRG2 expression in the colon cell lines. (A) In western blot analysis, E-cadherin expression was detected in Colo205, HCT116, LoVo and HT29 cell lines which also expressed NDRG2. (B) Modulation of NDRG2 was induced in the TGF- β -treated HCT116 cells and the E-cadherin expression was observed in those cells. (C) NDRG2 introduced KM12 cells were analyzed for E-cadherin expression.

In colorectal carcinoma, the variations of staining intensity were dependent on tumor differentiation. It was distinctively expressed in the well-differentiated colorectal carcinomas (Fig. 1B) compared to the less-differentiated tumor tissues (Fig. 1C). Notably, E-cadherin was more strongly expressed in the well-differentiated area than in the less differentiated area in the tumor tissue of each patient, similar to the expression pattern of NDRG2 as in our previous report (8). Statistical analysis showed a positive correlation between the expression status of NDRG2 and E-cadherin ($P < 0.0001$) (Table I).

Expression analyses of NDRG2 and E-cadherin in several colorectal cancer cell lines supported their positive relationships (Fig. 2A). Very low or no expression of NDRG2 and E-cadherin were detected in SW480, SW620 and KM12c cell lines. On the contrary, both NDRG2 and E-cadherin were expressed strongly in HCT116, Colo205, LoVo and HT29 cell lines. Moreover, the expression of NDRG2 was notably gradually decreased in the TGF- β -treated HCT116 cells in a dose-dependent manner. In these NDRG2 modulating conditions, the expression pattern of E-cadherin was similar to that of E-cadherin (Fig. 2B). The directly NDRG2 introduced SW480 cells also induced the E-cadherin expression (Fig. 2C).

NDRG2 regulates E-cadherin transcription via GSK-3 β activation. To explore the direct effect of NDRG2 on *E-cadherin* transcription, a reporter plasmid containing promoter region (-368~+51) of *E-cadherin* was used for luciferase assay. When NDRG2-expressing plasmid was co-transfected with the reporter plasmid, NDRG2 increased the *E-cadherin* promoter activity in a concentration-dependent manner (Fig. 3A). Introduction of NDRG2 into cell lines showing low level of NDRG2 expression induced the increase of *E-cadherin* promoter activity. Additionally, in the cell lines which express higher levels of NDRG2 and E-cadherin, downregulation of NDRG2 by siRNA treatment induced a decrease of *E-cadherin* promoter activity. Treatment of siNDRG2 decreased the *E-cadherin* promoter activity in a concentration-dependent manner in HCT116 and Colo205 cell lines (Fig. 3B). RT-PCR analysis confirmed that NDRG2 introduction into KM12 induced higher level of *E-cadherin* expression compared with control in which empty vector was transfected (Fig. 3C). Similarly, siNDRG2 treatment in HCT116 induced downregulation of *E-cadherin* transcription (Fig. 3D). Snail, a master regulator of EMT during embryonic development, was previously reported to be regulated by GSK-3 β activity (29,30).

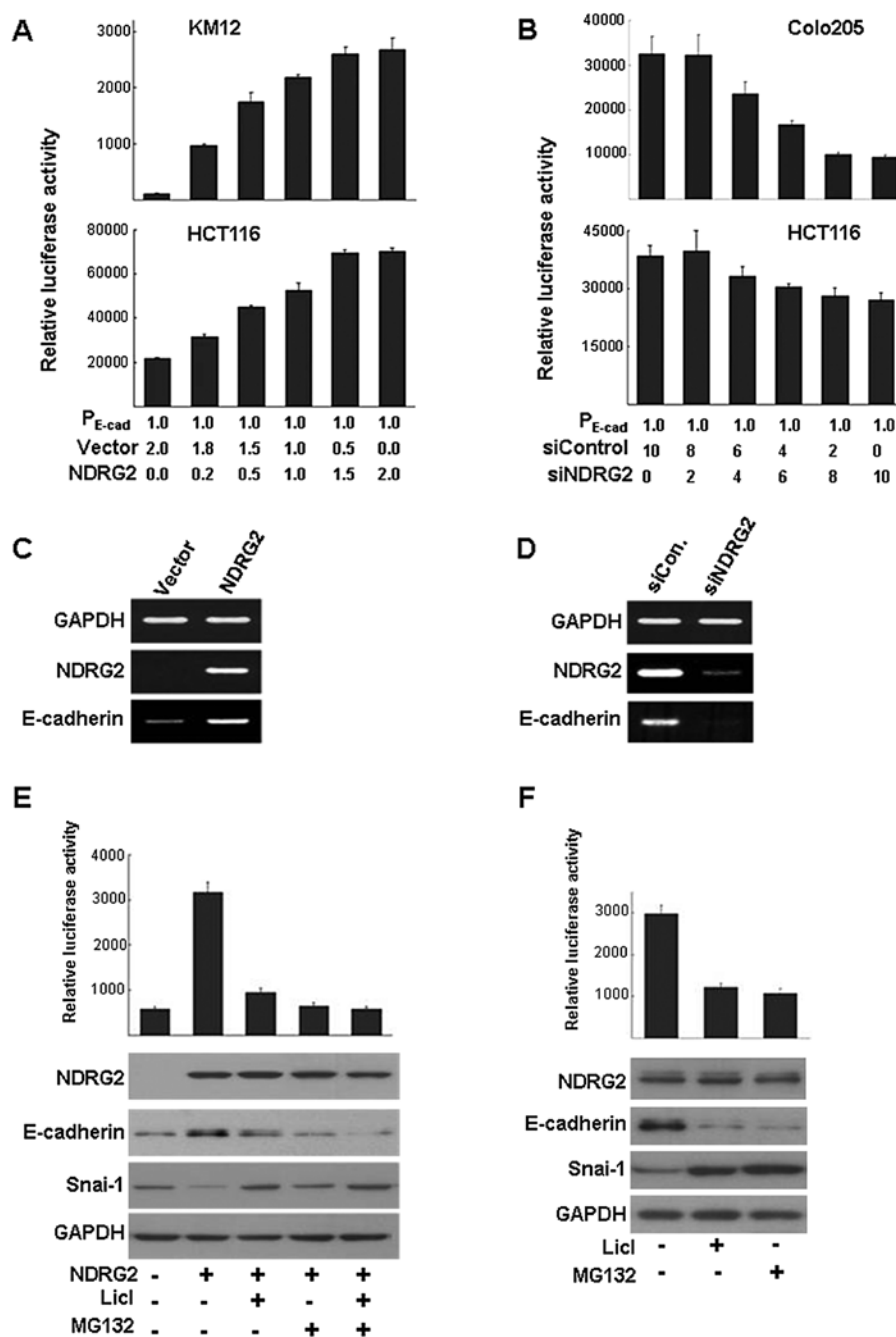


Figure 3. Transcriptional regulation of E-cadherin expression by NDRG2. (A) A reporter plasmid containing *E-cadherin* promoter region (-368~+51) was transfected into colon cancer cell lines with varying concentrations of NDRG2-expressing plasmid. The promoter activity was increased by NDRG2 expression in a concentration-dependent manner. Each data point was performed in triple, and the results were reported as mean absorption \pm standard error. (B) Small interfering RNA against *NDRG2* induced the decrease of *E-cadherin* promoter activity. (C) NDRG2-expressing plasmid was transfected into KM12c and its effect on E-cadherin expression was analyzed by RT-PCR. NDRG2 upregulation induced transcriptional increase of *E-cadherin*. (D) Downregulation of NDRG2 by siRNA treatment induced the decrease of *E-cadherin* transcription in the HCT116 cell line which expresses higher levels of NDRG2 and E-cadherin protein. (E) Inhibition of GSK-3 β activity by LiCl treatment or inhibition of proteasomal degradation by MG132 treatment blocked E-cadherin upregulating effect of NDRG2. (F) The treatment of LiCl or MG132 in the HCT116 cell line induced a decrease of E-cadherin expression.

In our previous study, we discovered that overexpression of NDRG2 in colon cancer cells induced GSK-3 β activation (8). We have studied the effect of NDRG2 on the regulation of Snai-1 (a human homologue of *Drosophila* Snail) expression through GSK-3 β activation. LiCl, a GSK-3 β inhibitor, and/or MG132, a proteasomal inhibitor, blocked NDRG2-mediated upregulation of E-cadherin expression even if there was a high expression level of NDRG2 (Fig. 3E). Transcription assay using reporter plasmid provided further evidence

that E-cadherin regulation by NDRG2 may be mediated by GSK-3 β . Downregulation of NDRG2 by siRNA treatment in HCT116 cells caused upregulation of Snai-1 expression and the decrease of E-cadherin (Fig. 3F).

Regulation of NDRG2 and E-cadherin expression by cell density. E-cadherin expression in HCT116 cells is regulated by cell densities (31). We verified the correlation between E-cadherin expression and cell density in HCT116 cells, and

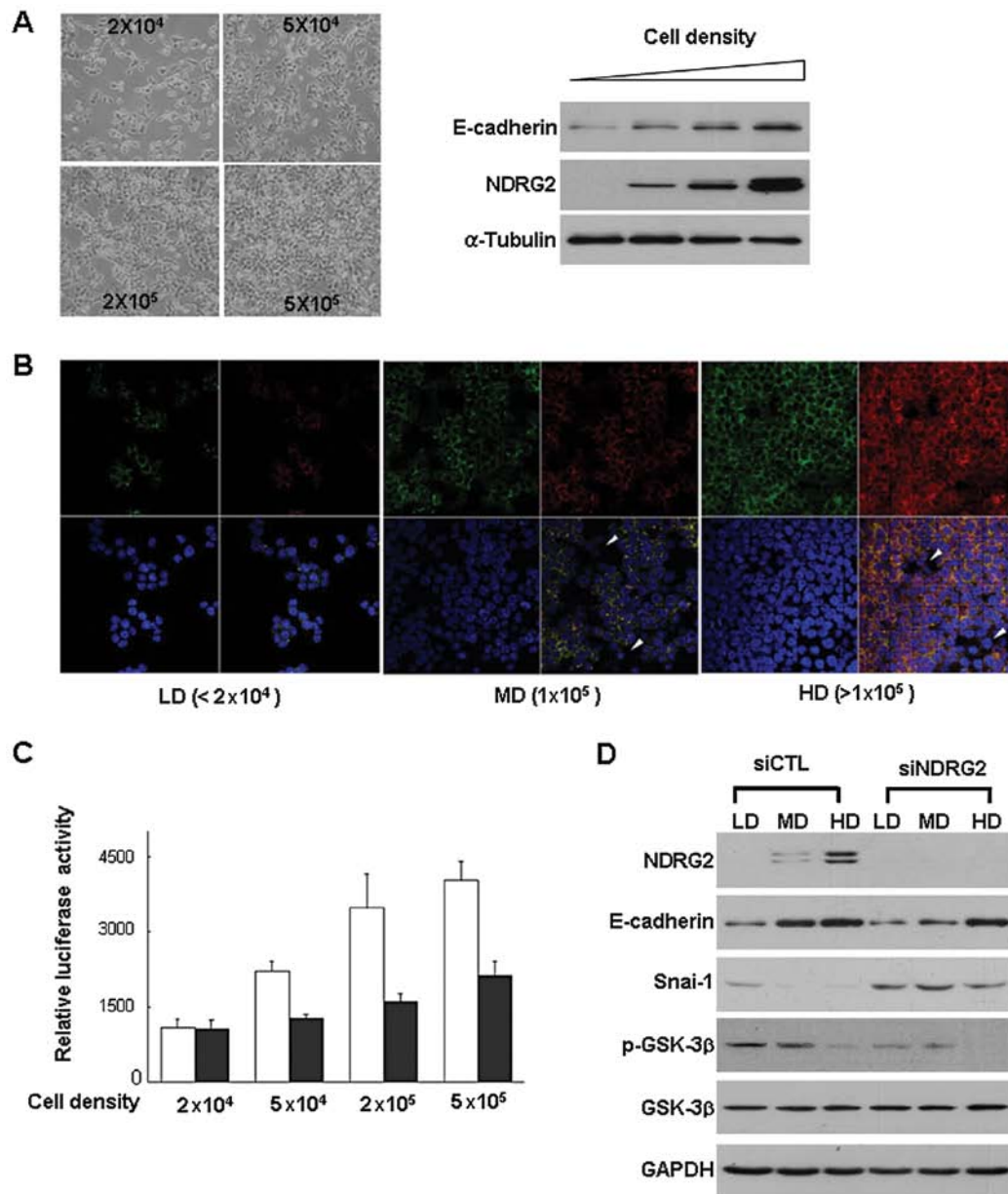


Figure 4. NDRG2 mediates density-dependent regulation of E-cadherin. (A) Both NDRG2 and E-cadherin expression levels were increased with cell density. In sparse conditions, NDRG2 was not detected and a very low level of E-cadherin expression was detected. On the contrary, NDRG2 was detected intensively at dense culture conditions and E-cadherin expression was also increased. (B) Confocal staining shows the correlation between NDRG2 and E-cadherin expression. In compact regions, strong signals for NDRG2 and E-cadherin were detected. Conversely, NDRG2 expression was rarely detected at lower density regions (arrow-head). LD, low density; MD, medium density; HD, high density. (C) *E-cadherin* promoter activity was increased with cell density. Knockdown of NDRG2 by siRNA treatment inhibited this effect of cell density. (D) NDRG2 expression was increased with cell density which was inversely correlated with phosphorylated GSK-3β. At a higher cell density, active form of unphosphorylated GSK-3β induced the downregulation of Snai-1 protein level followed by the increase of E-cadherin expression.

found that the amounts of E-cadherin and NDRG2 expression were increased with cell density concomitantly (Fig. 4A). This type of expressional correlation was verified by immunocytochemical data (Fig. 4B). E-cadherin staining exhibited the same patterns with NDRG2 staining which increased with cell density. In compact regions, both NDRG2 and E-cadherin were strongly detected. Conversely, NDRG2 expression was rarely detected at low-density regions (arrow-head) although total cell density was higher (Fig. 4B). Knockdown of NDRG2 blocked the density-induced increase of E-cadherin expression and *E-cadherin* promoter activity even in the highest cell density (Fig. 4C). As expected, phosphorylation level of

GSK-3β was decreased with cell density. However, siNDRG2 treatment did not induce any changes in GSK-3β status, indicating that GSK-3β activity can be regulated by NDRG2 function (Fig. 4D).

NDRG2 expression level is an independent prognostic factor for overall survival. We first carried out univariate analyses to examine whether the expression status of NDRG2 correlates with recurrence-free survival. A total of 38 patients (26.6%) presented with recurrence during the follow-up period. At the end of the follow-up, 81 (56.6%) patients were alive and 62 had died. The analysis showed that a high level of NDRG2 expres-

Table II. Multivariate Cox proportional hazards analysis for recurrence-free survival and overall survival.

	n	Recurrence-free survival		Overall survival	
		RR (95% CI)	P-value	Median (95% CI)	P-value
NDRG2 level					
Low/negative	77	1.000	0.051	1.000	0.018
High	66	0.572 (0.326-1.003)		0.486 (0.267-0.884)	
Age (years)					
<50	26	1.000	0.996	1.000	0.123
≥50	117	0.999 (0.554-1.799)		1.771 (0.856-3.663)	
Gender					
Female	68	1.000	0.132	1.000	0.680
Male	75	1.444 (0.895-2.330)		1.118 (0.658-1.899)	
Site					
Right colon	34	1.000	0.089	1.000	0.167
Left colon	109	1.696 (0.922-3.122)		1.614 (0.818-3.185)	
Size diameter (cm)					
<5	61	1.000	0.344	1.000	0.104
≥5	82	1.27 (0.774-2.085)		1.626 (0.905-2.922)	
Differentiation					
Well	34	1.000	0.612	1.000	0.933
Moderately	81	0.712 (0.360-1.409)		1.015 (0.467-2.205)	
Poorly	28	0.772 (0.319-1.873)		1.147 (0.444-2.964)	
Dukes' stage					
A and B	66	1.000	0.001	1.000	0.001
C and D	77	2.573 (1.507-4.395)		2.879 (1.585-5.230)	

P-values were obtained by Cox proportional hazards analysis modeled for the high and low/negative levels of NDRG2 expression. RR, relative risk.

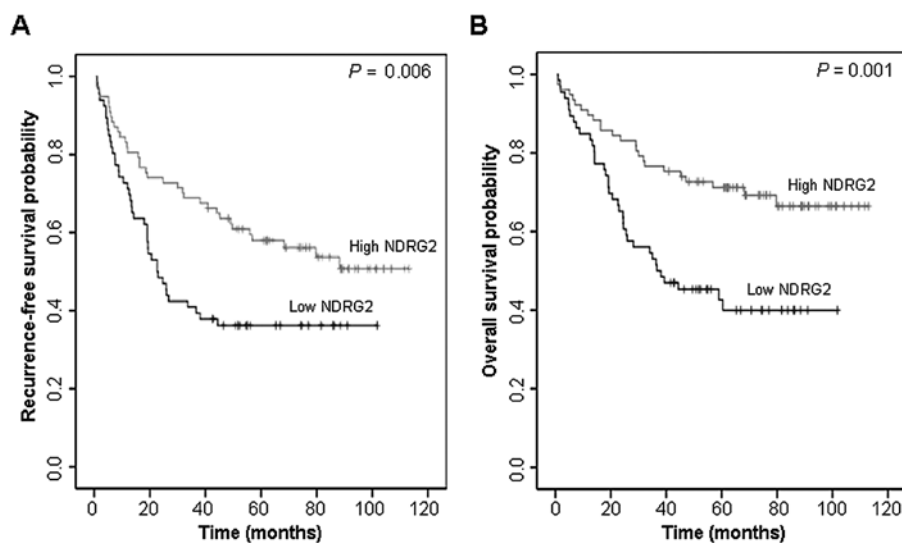


Figure 5. Kaplan-Meier survival analysis by NDRG2 expression status. (A) Cumulative recurrence-free survival differences between patients with high and low levels of NDRG2 expression. (B) Cumulative overall survival differences between patients with high and low levels of NDRG2 expression. P-value was obtained using the log-rank test of the difference.

sion was associated with recurrence-free survival ($P=0.006$), shown in Fig. 5A. A high level of NDRG2 expression also correlated significantly with favorable overall survival

($P=0.001$). Cumulative overall survival curves of patients were significantly split by NDRG2 expression status (Fig. 5B). The mean overall survival for patients with high and low level of

NDRG2 expression, and all patients were 84.9, 54.4 and 73.1 months, respectively. We carried out multivariate analyses to assess the predictive value of NDRG2 expression status for recurrence-free survival and overall survival by adjusting other potentially prognostic factors including age, gender, tumor site, tumor size, cell differentiation and tumor stage. The results corroborated a favorable survival outcome in patients with a high level of NDRG2 expression. In a multivariate Cox regression analysis, the independent prognostic factors significantly associated with overall survival were NDRG2 ($P=0.018$) and tumor stage ($P=0.001$). The relative risk (RR) of mortality was more than twice greater in patients with low NDRG2 (RR, 0.486; 95% CI, 0.267-0.884) than in those with high NDRG2. Although it did not reach statistical significance, a high level of NDRG2 expression was also predictive of increased recurrence-free survival with a P-value of 0.051. The relative risk (RR) of disease recurrence for patients with high NDRG2 was 0.572 (95% CI, 0.326-1.003). Results from the Cox proportional hazards analysis are summarized in Table II.

Discussion

Accumulating evidence presents the function of NDRG2 as a tumor suppressor active in early stages of tumor development; however, little is known about its molecular mechanisms. We previously discovered that NDRG2 inhibits tumor cell proliferation by modulating AP-1 transcription factor activity (32), and tumor cell metastasis by inactivating TCF/ β -catenin signaling (8). During these studies, we found that NDRG2 expression increases as cell density increases (Fig. 4A), which matches with E-cadherin expression pattern (31). This led us to study their expressional correlation and the functional relevance of NDRG2 during tumor metastasis.

We explored possible correlations between NDRG2 and E-cadherin expression status in colon cancer tissues (Fig. 1, Table I). Among 44 tumor tissues exhibiting a high level of NDRG2 immunoreactivity, 42 cases displayed high level of E-cadherin expression. On the contrary, 30 cases showed a correspondingly low level of E-cadherin among 55 tumor tissues showing low level of NDRG2. The likelihood of expressing high level of E-cadherin in tumors with high level of NDRG2 is 1.68 times (95% CI, 5.5416-114.5953) greater than in tumors with low levels of NDRG2. Indeed, the association between NDRG2 and E-cadherin expression status reached statistical significance based on the analysis ($P<0.0001$). This correlation in their expression patterns was confirmed by transfection experiments of NDRG2-expressing plasmid or siRNA into colon cancer cell lines.

Reporter assay and RT-PCR analysis indicated that NDRG2 regulates E-cadherin expression at the transcriptional level. E-cadherin is a key molecule in the maintenance of epithelial phenotype and its deregulation may be a critical step in EMT, which leads to metastasis of epithelial tumors. Transcriptional repression of *E-cadherin* and associated morphological changes in cells occur during EMT and some of these processes are related with Snail function regulated by Wnt/ β -catenin signaling (29,36-38). Snail, a zinc-finger transcriptional repressor, contains several consensus sites for GSK-3 β , and phosphorylation of these sites by GSK-3 β

facilitates its proteasomal degradation as β -catenin does (36). It was previously reported that NDRG2 functions as a tumor suppressor by regulating GSK-3 β signaling (8). We studied the functional relevance of GSK-3 β activity on the regulation of NDRG2 function to control E-cadherin expression. Increase of E-cadherin expression induced by NDRG2 was blocked by LiCl or MG132 treatment demonstrating that NDRG2 employs GSK-3 β kinase activity to regulate E-cadherin expression. Inhibition of proteasomal degradation mediated by GSK-3 β resulted in enhancement of Snail activity with concomitant downregulation of E-cadherin expression irrespective of the presence of NDRG2, which indicates NDRG2 is an upstream regulator of GSK-3 β -mediated E-cadherin regulation. GSK-3 β is also known as an endogenous inhibitor of Snail transcription (30). However, Snail downregulation by NDRG2 did not appear to be transcriptional in our system. mRNA level of *Snail* in colon cancer cell lines displayed no correlation with NDRG2 expression (Fig. 4D). Activated GSK-3 β also induces the degradation of β -catenin through proteasomal pathway. β -catenin known as a direct activator of Slug, a transcriptional repressor of *E-cadherin* (known as Snai-2 in human) (31). In our system, GSK-3 β -mediated modulation of β -catenin degradation was not detected (data not shown), as HCT116 is known to express degradation-resistant β -catenin mutant form (39,40).

Epithelial cells grow as clusters that maintain complete cell-to-cell adhesion and tightly packed structure is a major characteristic of epithelial tissues. In high-density culture conditions, cells will form compact layers assuming epithelial phenotype. On the contrary, cells will grow as mesenchymal structure in sparse culture conditions. Confocal staining of NDRG2 and E-cadherin showed these phenomena definitively (Fig. 4B). NDRG2 expression was increased with cell density and was the highest at the central region of dense culture in which each cell was surrounded by neighboring cells. However, at the marginal regions, NDRG2 expression was rarely detected (arrowhead in Fig. 4B). These expression patterns were the same for E-cadherin expression.

Recently, Piepoli *et al* (41) reported on NDRG2 promoter methylation during colorectal tumor development. However, we could not find a correlation between NDRG2 expression and promoter methylation status in colon cancer cell lines (data not shown). Instead, NDRG2 is known to be regulated by phosphorylation (32). According to western blot data, NDRG2 is shown as a doublet. Higher molecular weight band is the phosphorylated form of NDRG2 (Fig. 2A). Extracellular signals such as cytokines or hypoxic conditions can regulate NDRG2 phosphorylation status although we could not find the exact conditions for the phosphorylation. HIF-1 α may be a candidate for NDRG2 phosphorylation as it is activated by hypoxic conditions which occur during high density cell growth. A better understanding of the molecular mechanisms that control NDRG2 activity will help us to elucidate the early stage of tumor cell development.

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