Functional and Clinical Evidence for NDRG2 as a Candidate Suppressor of Liver Cancer Metastasis

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Abstract

We searched for potential suppressors of tumor metastasis by identifying the genes that are frequently down-regulated in hepatocellular carcinomas (HCC) while being negatively correlated with clinical parameters relevant to tumor metastasis, and we report here on the identification of N-myc downstream regulated gene 2 (NDRG2) as a promising candidate. NDRG2 expression was significantly reduced in HCC compared with nontumor or normal liver tissues (87.5% (35 of 40) and 62% (62 of 100) at RNA and protein levels, respectively). Reduction of NDRG2 expression was intimately associated with promoter hypermethylation because its promoter region was found to carry extensively methylated CpG sites in HCC cell lines and primary tumors. Immunohistochemical analysis of NDRG2 protein in 100 HCC patient tissues indicated that NDRG2 expression loss is significantly correlated with aggressive tumor behaviors such as late tumor-node-metastasis (TNM) stage (P = 0.012), differentiation grade (P = 0.024), portal vein thrombi (P = 0.011), infiltrative growth pattern (P = 0.015), nodal/distant metastasis (P = 0.027), and recurrent tumor (P = 0.021), as well as shorter patient survival rates. Ectopically expressed NDRG2 suppressed invasion and migration of a highly invasive cell line, SK-Hep-1, and experimental tumor metastasis in vivo, whereas small interfering RNA–mediated knockdown resulted in increased invasion and migration of a weakly invasive cell line, PLC/PRF/5. In addition, NDRG2 could antagonize transforming growth factor β–mediated tumor cell invasion by specifically down-regulating the expression of matrix metalloproteinase 2 and laminin 332 pathway components, with concomitant suppression of Rho GTPase activity. These results suggest that NDRG2 can inhibit extracellular matrix–based, Rho-driven tumor cell invasion and migration and thereby play important roles in suppressing tumor metastasis in HCC. [Cancer Res 2008;68(11):4210–20]

Introduction

Tumor metastasis and recurrence represent the most critical factors affecting the prognosis of cancer patients. However, most solid tumors suffer from the lack of therapies to control metastasis formation and tumor recurrence. Metastasis is a complex, multifaceted process involving tumor invasion, tumor cell dissemination, colonization of distant organs, and metastatic outgrowth (1–4). These processes are mediated by the genes regulating diverse molecular pathways such as cell invasiveness, motility, survival, proliferation, angiogenesis, and others (2–4). Efforts to define tumor metastasis in molecular terms have identified a number of metastasis-promoting genes, such as transforming growth factor (TGF)-β (5), vascular endothelial growth factor (6), and matrix metalloproteinases (MMP; ref. 7), as well as metastasis suppressors, such as KAI1 (8), NM23 (9), and KISS1 (10); yet, the understanding of the molecular mechanism underlying metastasis is far from complete.

Both the positive and negative regulators of tumor metastasis have a clinical significance as the diagnostic marker and/or target of therapeutic intervention. Positive regulators are usually up-regulated in metastatic cancers, and thus preferred over metastasis suppressors as the marker for predicting metastasis (4, 11). On the other hand, both inhibiting the positive regulators of metastasis and activating the metastasis suppressors are regarded as valid means of preventing metastasis formation because disruption of any step in the metastatic cascade could render metastatic cells nonmetastatic (4, 12).

Several different approaches have been used to identify metastasis suppressor genes (13). Typically, candidate metastasis suppressors were initially discovered from differential gene expression analyses [differential display (14), microarray (15), and subtractive hybridization (9)], genetic analysis [microcell-mediated chromosome transfer; ref. 16], clinical correlation analysis (17), or combinations thereof (10). They were then characterized in vitro and/or in vivo for their effects on invasiveness, motility, tumor growth, and metastasis.

As an attempt to identify novel metastasis suppressor genes having a clinical relevance with hepatocellular carcinoma (HCC), we searched for the genes frequently down-regulated in HCC that are also negatively associated with clinical parameters predictive of poor prognosis, such as recurrence and tumor grade. We identified the N-myc downstream regulated gene 2 (NDRG2) as one such candidate, and we provide in this report the biological evidence that it can function as a negative regulator of tumor cell invasion and migration and the clinical evidence that its down-regulation is associated with aggressive tumor behaviors and poor prognosis of HCC.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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References

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Materials and Methods

Reagents. TGF-β1 was obtained from R&D Systems, Inc. Synthetic small interfering RNAs (siRNA) for NDRG2 (sense, 5'-CUCACUCCUGCGGGAUAA-3' and antisense, 5'-UUAUCUCAGGAGAUGUATT-3') and green fluorescent protein (GFP; sense, 5'-GUUCAGCGUUGCGCGGAGTT-3' and antisense, 5'-CUCCGCGGACACGCGUGAATT-3') were purchased from Samchully Pharm Co., and EZ-Detect Rho activation kit was from Pierce, Inc. Monoclonal antibody (mAb) to NDRG2 protein was prepared from a hybridoma cell line established in-house by fusing the splenocytes of BALB/c mice immunized with recombinant NDRG2 protein with the SP2/0-Ag14 murine myeloma cells, and described in U.S. patent 11/398979.

Generation of stable transfectants overexpressing NDRG2. For overexpression of NDRG2, the coding region (nucleotides 150–1,222) of NDRG2 cDNA was inserted in-frame into pcDNA3.1/myc-his vector (Invitrogen). To prepare stable transfectants, SK-Hep-1 or PLC/PRF/5 cells were seeded in a six-well dish at 1 × 10⁵ per well and transfected with pcDNA3.1/myc-his vector containing NDRG2 cDNA or a control vector using Lipofectamine Plus reagent (Invitrogen). After culturing in complete medium containing G418 (Invitrogen) for 2 weeks, individual colonies were isolated. For in vivo metastasis experiments, SK-Hep-1 cell lines stably overexpressing NDRG2 were established in a murine myeloma cell line and described in U.S. patent 11/3938979. Inc. Monoclonal antibody (mAb) to NDRG2 protein was prepared from a hybridoma cell line established in-house by fusing the splenocytes of BALB/c mice immunized with recombinant NDRG2 protein with the SP2/0-Ag14 murine myeloma cells, and described in U.S. patent 11/398979.

Reporter assay. Cells were seeded at a density of 5 × 10⁴ per well in 12-well plates and transfected with 0.5 μg DNA (0.4 μg 3TP-Luc and 0.1 μg renilla-Luc) per well using Lipofectamine Plus reagent according to the supplier's instruction (Invitrogen). Luciferase activity was measured using the luciferase assay system (Promega) in a luminometer (Lumat LB9501, Berthold). Renilla luciferase was used as the internal control for normalization of transfection efficiency. Luciferase assays were done in duplicates, and each experiment was repeated at least twice.

Tissue arrays and immunohistochemical analysis. For tissue array, surgically resected samples from patients with pathologically defined HCC were collected by Inje University Seoul Paik Hospital, Seoul, Korea. All tissues were routinely fixed in 10% buffered formalin and embedded in paraffin blocks. Contents of the experiments involving human tissue specimen were reviewed and approved by the Internal Review Board. Core tissue biopsies (2 mm in diameter) were taken from individual paraffin-embedded HCC (donor blocks) and arranged in a new recipient paraffin block (tissue array block) using a microtome apparatus (Superchips Laboratories). The resulting tissue array blocks (TA 128 and TA129) contained 100 HCCs and 20 nonneoplastic liver tissues. Four-micrometer-thick sections of the tissue array blocks were subjected to immunohistochemical study. Following deparaffinization and antigen retrieval, the slides were labeled with a mAb to NDRG2. Labeling was detected using the avidin-biotin complex (ABC) method. 3',3'-Diaminobenzidine was used as a chromogen. Normal saline was used as a substitute for the primary antibody for the negative control reaction. NDRG2 expression was scored as positive if ≥10% of the cells showed moderate to strong staining, weak if either cytoplasmic or membranous staining was noted in <10%, and negative if neither cytoplasmic nor membranous staining was seen. For the statistical analysis of the tissue array data, categorical variables were compared using the χ² test, and analyzed with SPSS statistical software version 11.01. Survival rates were compared by Kaplan-Meier test (for univariate analysis) and Cox proportional hazards regression (for multivariate analysis) using the same software. P < 0.05 was regarded as significant.

Reverse transcription-PCR. Reverse transcription-PCR (RT-PCR) experiments were carried out according to the standard protocol. The sequences of PCR primers are described in Supplementary Table S1. The optimized PCR conditions were as follows: 1 cycle at 95°C for 30 s; 30 cycles (semiquantitative) or 45 (real-time) cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 10 min. Specificity of the real-time RT-PCR amplification was checked by a melting curve analysis (from 50°C to 90°C) using SYBR premix Ex Taq (TaKaRa Bio) in Exicycler (Bioneer). Relative gene expression levels were calculated as 2−ΔΔCT after normalization against β-actin, where C_t was defined as the threshold cycle number of PCR at which amplified product was first detected.

Western blotting. Cells were lysed in a lysis buffer [1% Triton X-100, 150 mM NaCl, 100 mM KCl, 20 mM HepES (pH 7.9), 10 mM NaCl, EDTA, 1 mM sodium orthovanadate, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mM phenylmethanesulfonyl fluoride] on ice. Western blot analyses were carried out according to the standard protocol using nitrocellulose membranes (Bio-Rad). For immunoblotting, membranes were incubated with the primary antibody (1:5,000) for 2 h, followed by 1-h incubation with a 1:5,000 dilution of horseradish peroxidase (HRP)-linked secondary antibody. Finally, the immunoreactive proteins were detected by enhanced chemiluminescence assay with HRP (Pierce).

Bisulfite sequencing and methylation-specific PCR. Genomic DNA modification with sodium bisulfite was done as previously reported (18). The bisulfite-modified DNA was resuspended in 30 μL of sterile water, and 10 nanograms were amplified in 5% DMSO, 20 mM Tris-HCl (pH 8.8), 2 mM MgCl₂, 10 mM Tris-HCl, 1.25 mM deoxyribonucleotide triphosphates, 400 mM/L of primer pair, and 5 units of Taq DNA polymerase (Roche). Cycling conditions were as follows: preincubation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s, and a final extension of 5 min at 72°C. PCR products were purified and cloned into pGEM-T-Easy Vector System (Promega) for DNA sequencing analysis. Primer sequences for the bisulfite sequencing and methylation-specific PCR of the NDRG2 promoter region are described in Supplementary Table S1. PCR reactions using the GampampTHI primer set was carried out at the following conditions: hot start at 95°C for 6 min, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. The PCR conditions for methylated primer set were the same except for the annealing temperature (56°C).

Gelatin zymography. Gelatin zymography was carried out to determine the activities of MMP2 and MMP9 expressed in mock- or NDRG2-transfected PLC/PRF/5 cells after stimulating them with TGF-β1 (5 ng/mL) for 48 h. Briefly, spent culture media were loaded to 10% SDS-polyacrylamide gel containing 1 mg/mL gelatin (Sigma) under nonreducing conditions. Following electrophoresis, the gels were washed twice with 2.5% Triton X-100 (Sigma) to remove SDS and then incubated in a zymographic buffer [0.5 mol/L Tris-HCl (pH 7.5), 50 mM NaCl, 2 mol/L NaCl] overnight at 37°C. Gels were stained with 0.05% Coomassie brilliant blue R-250. Gelatinase activity was visualized as clear bands against the blue-stained gelatin background.

Migration and invasion assays. Cell migration and invasion assays were done essentially as previously described (19). For migration assay, the under surface of Transwell filter inserts (Corning Costar) was precoated with fibronectin at a concentration of 20 μg/mL. The Transwells were then assembled in a 24-well plate, with the lower chamber filled with 800 μL of serum-free medium and the upper chamber inoculated with 200 μL of cells (5 × 10⁴/mL). For the invasion assay, cells (5 × 10⁴/mL) were seeded onto Transwell filters coated with Matrigel (upper side of the filter) and fibronectin (underside of the filter). The Transwell was then left in a CO₂ incubator for 24 h. Cells that invaded into the lower surface of the filters were fixed with methanol and stained with H&E (Sigma-Aldrich). The number of migrated or invaded cells per microscopic field was counted at ×50 magnification.

Metastasis analysis in a mouse model. In vivo metastasis experiments were done using 4-wk-old female BALB/c nu/nu mice (Japan SLC). Mice were anesthetized by inhalation with diethyl ether, and spleens exteriorized.
via a flank incision. SK-Hep-1 cells mock infected or infected with NDRG2-lenti virus (1 x 10⁶ in 100 μL) were injected into the lower polar side of spleen through a 27-gauge needle, and splenectomies done 1 min later. All animals were sacrificed at 4 wk, and the metastatic nodules in the liver counted.

Results

NDRG2 expression is significantly reduced in HCC. To identify the potential suppressors of tumor metastasis in HCC, we first analyzed the gene expression profiles of 57 tumor and 41 nontumor liver tissues of HCC patients using cDNA microarrays (total 49K), and identified the genes that are frequently down-regulated in HCC by significance analysis of microarray (unpaired two-class; Supplementary Table S2; ref. 20). We then estimated the potential clinical significance of these genes by correlating their expression pattern with the clinical parameters relevant to tumor metastasis (Supplementary Table S3). Based on the reasoning that potential metastasis suppressors rank high in terms of the degree and the clinicopathologic significance of down-regulation in tumors, we chose NDRG2 as one of the best candidates fulfilling these criteria (Fig. 1A; Supplementary Tables S2 and S3). The top three most down-regulated genes, SLC22A3, LCAT, and LOC401022, were not selected for further consideration because they were much less significantly associated with the clinicopathology of HCC than NDRG2 (Supplementary Table S3) and less likely to play regulatory roles in tumor metastasis according to their known functions or the functional structure of the predicted protein product (data not shown). In contrast, NDRG2 exhibited an extensive negative correlation with various clinical features of HCC progression, such as metastasis (P = 0.01167 for extrahepatic), recurrence (P = 0.0084), invasion (P = 0.00161–0.01887 for various forms of invasion), extranodular growth (P = 0.00927), tumor size (P = 0.00601), and Edmondson's tumor grade (P = 0.00023; Supplementary Table S3). Moreover, its amino acid sequence is highly related to that of NDRG1 (57% homology), which is known to have roles in stress responses, cell proliferation and differentiation, and suppression of tumor metastasis (14, 21, 22). We carried out a real-time RT-PCR analysis for 20 paired tumor-nontumor samples from the HCC patients who had undergone partial hepatectomy, and confirmed that NDRG2 expression was significantly reduced in
recurrent tumors ($P = 0.00775$) and advanced histologic grades ($P = 0.02142$; Fig. 1B). We also found from a Western blot analysis that the NDRG2 protein levels in recurrent and high-grade tumors were mostly lower than those in nonrecurrent and low-grade tumors (Fig. 1C). Therefore, we conclude that NDRG2 expression is significantly down-regulated in HCC at both mRNA and protein levels in a manner negatively associated with aggressive tumor behaviors.

**Evaluation of the clinical significance of NDRG2 by immunohistochemical analysis of liver cancer tissues.** We extended our analysis on the clinical significance of NDRG2 by immunohistochemically examining the protein expression in a larger number of HCC tissues collected from a cohort distinct from the one used for cDNA microarray analysis. In a preliminary analysis with nonneoplastic liver tissues, NDRG2 protein was found to be expressed in the cytoplasm and plasma membrane of hepatocytes and bile duct epithelial cells with a moderate to strong staining intensity (Fig. 2 A, a). In tumor cells, NDRG2 showed variable expression patterns, comparable to or much weaker than its level in nonneoplastic liver tissues or not detectable at all (Fig. 2 A, b and c). Interestingly, in the boundary between HCC and nonneoplastic liver tissues, NDRG2-negative HCC cells often seemed to be infiltrating into adjacent nontumor tissues expressing NDRG2 (Fig. 2 A, d).

The clinicopathologic significance of NDRG2 down-regulation in HCC was evaluated by correlating its expression pattern with the clinicopathologic characteristics of HCC patients. The expression pattern of NDRG2 was evaluated in formalin-fixed paraffin-embedded liver tissues from 100 HCC patients who were alive and had died during the follow-up period. Immunohistochemistry was performed using the ABC method with a 1:250 dilution of primary antibody to NDRG2. The staining results were evaluated microscopically by two pathologists, and the correlation between the intensity of staining and the clinical significance was analyzed using the Cox regression model. The incorporated clinical variables were selected from the factors showing prognostic value by univariate analysis. Variables such as tumor size, portal vein thrombi, intrahepatic metastasis, and lymph node/distant metastasis were not included because they already played a role in defining TNM stage (American Joint Committee on Cancer, http://www.cancerstaging.org/). *, liver functional status: B/C ($n = 13$) versus A ($n = 87$).
We next examined whether NDRG2 down-regulation is correlated with HCC patient survival and found that the "negative/weak" group had significantly less favorable overall survival rates than the "positive" group ($P = 0.017$; Fig. 2B). A multivariate analysis including NDRG2 expression status, patient's age, TNM stage, histologic grade, growth pattern, and liver functional status (Child-Pugh stage) also revealed that NDRG2 expression loss was a statistically significant factor in predicting poor overall survival (hazard ratio, 2.390; $P = 0.038$) but next only to TNM stage and Child-Pugh stage (Fig. 2C; Supplementary Table S4).

These results confirm that NDRG2 down-regulation is associated with advanced and aggressive tumor behaviors that are relevant to tumor metastasis and survival in HCC.

### Methylation pattern of the CpG sites of NDRG2 promoter

To understand the molecular mechanism of the predominant transcriptional repression of NDRG2 in HCC, we investigated the DNA methylation status of a CpG-rich region encompassing the proximal promoter and exon 1 of NDRG2 (−421 to +100 nucleotides) through bisulfite sequencing and methylation-specific PCR (Fig. 3A). Faithful cytosine-thymine conversion was confirmed from the bisulfite sequencing data by the lack of cytosine residues in non-CpG sites in HepG2 cells and normal liver tissue (Fig. 3B, top). Several methylated CpG sites were detected within the NDRG2 promoter in HepG2 cells but not in the normal liver tissue. Hypermethylation of the NDRG2 promoter region was also observed in SK-Hep-1, SNU-354, and SNU-368 cell lines (Fig. 3B, bottom). The methylated CpG sites were highly concentrated in CpG #1–16 in all the cell lines examined, allowing the design of a methylation-specific PCR primer set for a simpler detection of promoter hypermethylation. Further methylation-specific PCR analysis using the primer set shown in Fig. 3A revealed that Hep3B, PLC/PRF/5, and SNU-182 cell lines were methylated at the NDRG2 promoter region (Fig. 3C, top). RT-PCR analysis showed that NDRG2 mRNA expression is much repressed in these hypermethylated cell lines compared with normal liver (Fig. 3C, bottom). These results indicate that the transcriptional repression of NDRG2 in liver cancer cell lines is highly correlated with promoter hypermethylation.

To determine whether the aberrant hypermethylation of the NDRG2 promoter region also occurs in primary HCC tissues, we investigated the DNA methylation status in nine paired tumor-nontumor tissues by methylation-specific PCR. The NDRG2 promoter sequences were frequently hypermethylated in tumor tissues in contrast to the nontumor tissue of the same individual (7 of 9 cases; Fig. 3D, top). Some methyl CpG sites were also detected in nontumor tissues but the frequency was much lower than in tumor tissues. We also found from a RT-PCR analysis that the expression pattern of NDRG2 mRNA in HCC tissues was inversely related to the extent of promoter methylation (Fig. 3D, bottom). These findings indicate that promoter hypermethylation of NDRG2 is tumor specific and represents one of the major mechanisms for silencing NDRG2 expression in HCC.

NDRG2 can suppress tumor cell invasion and migration. The expression pattern of NDRG2 in HCC and its association with clinical parameters relevant to malignant tumor progression and recurrence prompted us to explore its possible role in tumor

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**Table 1. Clinicopathologic features of HCC in relation to the NDRG2 expression pattern**

<table>
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<tr>
<th>Clinicopathologic parameter</th>
<th>NDRG2 expression*</th>
<th>$P$</th>
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<tr>
<td></td>
<td>Negative/weak</td>
<td>Positive</td>
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<tr>
<td>Age (y)</td>
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<tr>
<td>Young (≤53)</td>
<td>32</td>
<td>15</td>
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<tr>
<td>Elderly (≥54)</td>
<td>30</td>
<td>23</td>
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<tr>
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</tr>
<tr>
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<td>35</td>
</tr>
<tr>
<td>Female</td>
<td>11</td>
<td>3</td>
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<tr>
<td>Tumor size</td>
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<td></td>
</tr>
<tr>
<td>Small (≤5)</td>
<td>30</td>
<td>21</td>
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<tr>
<td>Large (≥5)</td>
<td>32</td>
<td>17</td>
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<tr>
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<tr>
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<td>24</td>
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<tr>
<td>III-IV</td>
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<td>14</td>
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<td>Edmondson grade</td>
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<tr>
<td>Grade 3/4</td>
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<td>22</td>
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<td>Portal vein thrombi</td>
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<td>Intrahepatic metastasis</td>
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<td>Present</td>
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<td>Lymph node or distant metastasis</td>
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<td>46</td>
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<tr>
<td>Present</td>
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<tr>
<td>Recurrent HCC</td>
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<tr>
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* NDRG2 expression was determined by immunohistochemical staining of HCC tissues on a tissue microarray using a mAb to NDRG2.

† The stage was determined by the TNM staging criteria of the 6th edition of American Joint Committee on Cancer (http://www.cancerstaging.org/).
metastasis. We first examined the effect of NDRG2 on tumor cell invasion in two different liver cancer cell lines, PLC/PRF/5 and SK-Hep-1, which were reported to be weakly and highly invasive, respectively (23). As shown in Fig. 4A, forced expression of NDRG2 in these cell lines significantly suppressed their invasiveness through Matrigel ($P < 0.01$). We also found that NDRG2 could significantly suppress the migration of both cell lines ($P < 0.01$; Fig. 4B). On the other hand, siRNA-mediated knockdown of NDRG2 increased the motility and invasiveness of PLC/PRF/5 cells to a significant level (Fig. 4C). The antimigratory activity of NDRG2 was confirmed by a wound healing assay in SK-Hep-1 cells that stably express NDRG2 (data not shown).

We next examined the inhibitory effect of NDRG2 on the ability of liver cancer cells to colonize the liver after entry into hepatic portal system in vivo, using the splenic injection model (24). SK-Hep-1 cells injected into the spleen of nude mice metastasized to the liver and formed a multitude of white colonies visible from the surface (Fig. 4D). However, ectopic expression of NDRG2 in these cells significantly reduced their colonization in the liver, suggesting that NDRG2 can inhibit the metastasis formation of SK-Hep-1 cells at a distant site in vivo. This result, together with those of in vitro cell invasion and migration assays, indicates that NDRG2 may be a novel suppressor of tumor metastasis in HCC.

NDRG2 can antagonize TGF-$eta$1 in inducing tumor cell invasion. TGF-$eta$1 is known to play pivotal roles in promoting tumor cell invasion and metastasis and is overexpressed in advanced cancers including HCC (25–27). We therefore examined

Figure 3. Methylation pattern of the promoter region of NDRG2 in HCC. A, schematic map of 32 CpG sites (vertical lines) in the promoter region of the NDRG2 gene. The CpG sites were numbered from 421 bases upstream of the transcription initiation site (+1). The positions of methylation-specific PCR primers encompassing five CpG sites are indicated by arrows (forward arrows, CpG 4, CpG 5, and 6; reverse arrows, CpG 11 and CpG 12). B, bisulfite sequencing of the NDRG2 promoter region in normal liver tissue and liver cancer cell lines. Top, raw sequencing graphs for the bisulfite-treated NDRG2 promoter region. The original CpG sites are underlined. Bottom, summarized results for bisulfite sequencing analysis of the NDRG2 promoter in normal liver and various liver cancer cell lines. Each circle indicates a CpG site: open circle, unmethylated CpG; solid circle, methylated CpG. Numbers correspond to the position of CpG sites shown in A. C, methylation-specific PCR analysis of the NDRG2 promoter in liver cancer cell lines (top) and the corresponding RT-PCR data for the NDRG2 mRNA expressed in each cell line (bottom). The position of methylation-specific PCR primer set is indicated in A. U, unmethylated; M, methylated. D, methylation-specific PCR analysis of the NDRG2 promoter in nine pairs of tumor (T) and nontumor (NT) tissues of HCC (top two rows) and the corresponding expression pattern of NDRG2 mRNA as determined by RT-PCR (last row). Methylation-specific PCR primers are described in A. Normal liver was included as the control.
The regulatory relationship between TGF-β1 and NDRG2. TGF-β1 suppressed NDRG2 mRNA expression in PLC/PRF/5 cells while augmenting the expression of the invasion/motility-inducing gene plasminogen activator inhibitor type 1 (PAI-1; Fig. 5A, left). This was also observed in HepG2 and HaCaT cell lines (data not shown). In turn, NDRG2 inhibited the TGF-β1-mediated induction of PAI-1 expression. In a parallel experiment, we examined the effect of NDRG2 on the TGF-β1 modulation of 3TP promoter, which was...
derived from the PAI-1 gene promoter and contained multiple copies of TGF-β responsive elements (28), and found that its activity was significantly repressed by NDRG2 in PLC/PRF/5 cells (Fig. 5A, right). We then examined the effect of NDRG2 on TGF-β1-induced tumor cell invasion in PLC/PRF/5 cells and found that NDRG2 could inhibit the TGF-β1 action in promoting tumor cell invasion (Fig. 5B).

To understand the molecular basis of the NDRG2 antagonism of TGF-β1 action, we analyzed the effect of NDRG2 on the expression of the genes involved in tumor cell invasion in TGF-β1-stimulated PLC/PRF/5 cells. TGF-β1-mediated expression of epithelial-mesenchymal transition regulators such as E-cadherin, snail, and slug was not changed by NDRG2 (data not shown). However, NDRG2 significantly suppressed the TGF-β1-mediated induction of MMP2, an extracellular matrix (ECM)-degrading enzyme that plays roles in tumor cell invasion and activation of latent extracellular TGF-β (ref. 7; Fig. 5C, top). We also examined the biochemical activity of MMPs by gelatin zymography and found that the culture supernatant of NDRG2-overexpressing cells exhibited much weaker gelatinase activity compared with control (Fig. 5C, bottom). We then analyzed the consequences of NDRG2 expression on the laminin 332 (formerly known as laminin 5) pathway, which is known to play critical roles in tumor cell invasion, migration, and survival during metastasis (29). NDRG2 suppressed the TGF-β1 induction of β3 (LAMB3) and γ2 (LAMC2) subunits of laminin 332 and α3 chain (ITGA3) of α3β1 integrin (Fig. 5D, left). Because the activation of Rho GTPase activity by the engagement of α3β1 integrin with laminin 332 is crucial for tumor cell invasion (29), we examined the effect of NDRG2 on Rho GTPase activity and found that both the basal and TGF-β1-induced levels of GTP-bound, active Rho protein were decreased by NDRG2 (Fig. 5D, right). These results indicate that NDRG2 can block the Rho-driven tumor cell invasion process mediated by TGF-β1 by suppressing the induction of the genes involved in ECM degradation and ECM-based cell motility.

**Figure 5.** NDRG2 suppresses TGF-β1-induced tumor cell invasion. A, effect of NDRG2 on TGF-β1–induced gene expression. Mock- or NDRG2-overexpressing PLC/PRF/5 cells were stimulated with TGF-β1 (5 ng/mL) for 24 h, and the expression pattern of PAI-1 was analyzed by RT-PCR (left). Right, effect of NDRG2 on the TGF-β1 modulation of 3TP-Luc reporter. Firefly lucerase activity was measured from the extracts of PLC/PRF/5 cells treated with the indicated amount of TGF-β1 for 24 h. Renilla lucerase reporter was used as the internal control for transfection efficiency. Points, mean of three independent experiments; bars, SE. B, effect of NDRG2 on TGF-β1–induced invasion of PLC/PRF/5 cells. The invasion ability of nonstimulated or TGF-β1 (5 ng/mL; 24 h)–stimulated cells was estimated using Transwell filters coated with Matrigel (upper side) and fibronectin (underside). *P < 0.05, versus mock (t test). C, effect of NDRG2 on the TGF-β1 modulation of gelatinase activities. Mock-transfected or NDRG2-overexpressing PLC/PRF/5 cells were stimulated with TGF-β1 (5 ng/mL) for 24 h, and the mRNA expression of gelatinase A (MMP2) and gelatinase B (MMP9) genes analyzed by RT-PCR (top). Bottom, gelatin zymograms measuring the biochemical activity of gelatinases in the corresponding culture supernatant. D, effect of NDRG2 on the TGF-β1 modulation of the laminin 332 pathway. PLC/PRF/5 cells were treated with TGF-β1 as described in C, and the expression pattern of laminin 332 pathway components was analyzed by RT-PCR (left). Right, effect of NDRG2 on the activity of Rho GTPase, a critical intracellular signaling component of the laminin 332 pathway. The Rho GTPase activity was measured as the level of GTP-bound Rho protein (Rho-GTP) in the immunoprecipitated extracts of mock-transfected or NDRG2-overexpressing PLC/PRF/5 cells. TGF-β1 stimulation was done at 5 ng/mL for 15 min and immunoblotting was carried out with anti-Rho antibody. Levels of total Rho protein in total cell extracts are shown as a loading control.
Discussion

In this study, we investigated the biological function and clinical significance of NDRG2 in HCC progression. We found that NDRG2 was frequently down-regulated in HCC and provided evidence suggesting that this observation can have significant implications in the negative regulation of tumor metastasis.

Several pieces of evidence suggest that NDRG2 might be a novel candidate suppressor of tumor metastasis in HCC. First, down-regulation of NDRG2 expression is frequently observed in primary tumors and tumor cell lines. Our data showed that NDRG2 expression was significantly reduced in the majority of HCCs compared with adjacent nontumor tissues or normal liver. Moreover, NDRG2 remained down-regulated in the secondary tumors formed during the metastatic progression of HCC (Supplementary Fig. S1). Loss of NDRG2 expression has also been reported in meningioma and glioma tissues and glioblastoma cell lines (30, 31). Second, the loss of NDRG2 expression is significantly associated with malignant tumor features with increased metastatic potential and poor prognosis. Immunohistochemical analysis of NDRG2 protein expression in HCC by tissue microarray indicated that NDRG2 expression loss is closely correlated with portal vein thrombi, infiltrative growth pattern, nodal/distant metastasis, and recurrence. We also found that recurrent tumors derived from intrahepatic invasion exhibited significantly lower levels of NDRG2 mRNA expression than those of multicentric origins (P = 0.0032; Supplementary Fig. S2). Previous reports showed that reduced expression of metastasis repressors such as NM23 and KAI1 could be correlated with poor prognosis of tumors (32, 33), and we found in the present study that NDRG2 down-regulation is one of the independent poor prognostic factors by multivariate survival analysis. Third, our functional studies showed that NDRG2 can inhibit tumor cell invasion and migration on reexpression or ectopic expression in highly invasive tumor cell lines in vitro and in vivo. It could also antagonize TGF-β1-mediated tumor cell invasion and migration with concomitant down-regulation of molecular markers involved in ECM degradation and ECM-based cell motility. Finally, NDRG2 has an amino acid sequence highly related to that of its homologue NDRG1 (57% homology; ref. 22), which has been identified as a candidate suppressor of metastasis in colon and prostate cancers (14, 21). Together, these data indicate that NDRG2 might have a clinical significance as a marker associated with negative regulation of tumor metastasis in HCC.

The loss of NDRG2 expression could have been caused by a number of genetic or epigenetic events during hepatocarcinogenesis. Frequent losses of chromosomes 1p, 4q, 6q, 8p, 13q, 16q, and 17p have been reported in HCC, but the loss of 14q, where the NDRG2 and 17p have been reported in HCC, but the loss of 14q, where the NDRG2 candidate suppressor of tumor metastasis in HCC. First, down-regulation of molecular markers involved in ECM degradation and ECM-based cell motility. Finally, NDRG2 has an amino acid sequence highly related to that of its homologue NDRG1 (57% homology; ref. 22), which has been identified as a candidate suppressor of metastasis in colon and prostate cancers (14, 21). Together, these data indicate that NDRG2 might have a clinical significance as a marker associated with negative regulation of tumor metastasis in HCC.

The loss of NDRG2 expression could have been caused by a number of genetic or epigenetic events during hepatocarcinogenesis. Frequent losses of chromosomes 1p, 4q, 6q, 8p, 13q, 16q, and 17p have been reported in HCC, but the loss of 14q, where the NDRG2 gene is located, was rarely observed (34). Therefore, the major cause of NDRG2 expression loss in HCC could be either changes in trans-acting factor activity or methylation-induced promoter inactivation. Down-regulation of NDRG2 by N-Myc, as its name implies, has not been reported yet, nor has there been any report on the up-regulation of N-Myc in human HCC. Therefore, N-Myc-mediated regulation is not likely a common mechanism of NDRG2 down-regulation. Recently, c-Myc-mediated repression has been shown in HeLa and HEK293 cell lines (35). However, we did not find a significant negative correlation between the mRNA levels of c-Myc and NDRG2 in HCC tissues (Supplementary Fig. S3). In the present study, we showed that the CpG sites in the NDRG2 promoter were mostly hypermethylated in liver cancer cell lines and primary tumors, whereas those in normal liver tissues remained unmethylated. Loss of NDRG2 expression due to promoter hypermethylation has also been reported in meningioma (30) although the position of CpG methylation sites was somewhat different from what we observed in HCC. We also found that the loss of NDRG2 expression in primary HCC is carried on to the secondary tumors formed during metastatic progression, likely due to inheritance of the promoter hypermethylation pattern at NDRG2 locus (Supplementary Fig. S1). Therefore, we conclude that methylation-induced promoter inactivation might be the major cause of the frequent loss of NDRG2 expression in HCC. However, for the other cases, NDRG2 expression loss could be due to changes in trans-acting factor activities.

Our data provided the first functional evidence that NDRG2 can inhibit the invasion and migration of hepatoma cells. HCC is known as an invasive cancer, with liver itself being the most frequent target of metastasis (36–39). However, in HCC, tumor cells grow embedded in a microenvironment with a high content of ECM proteins, such as laminin, collagen, vitronectin and fibronectin, as a consequence of the development of cirrhosis (40). Therefore, crossing the ECM barriers by tumor cells is regarded to be particularly important for metastatic progression of HCC. Among the many different categories of molecules regulating tumor metastasis, we focused on the effect of NDRG2 on the expression of the genes responsible for tumor invasion such as epithelial-mesenchymal transition regulators, ECM proteins and their cell-surface receptors, and ECM-degrading enzymes. We found that NDRG2 can antagonize the function of TGF-β1 in promoting tumor invasion and migration by abrogating the induction of gelatinase activity and laminin 332 pathway. Gelatinases (MMP2 and MMP9) are important players of tumor invasion that degrade ECM components and activate the latent TGF-β present in extracellular space (7, 41). The laminin 332 pathway is also known to play critical roles in tumor cell invasion, migration, and survival during metastasis (42, 43). The proteolytic processing of laminin 332 γ2 chain via MMP2 exposes a cystic promigratory site on laminin 332, providing a trigger for cell migration and invasion (44, 45). Laminin 332 is expressed do novo in HCC, absent in the peritumoral tissues (23), and differentially expressed in metastatic and nonmetastatic HCCs (46, 47). Major extracellular components of the laminin 332 pathway include laminin 332, integrins α3β1 and α6β4, collagen XVII, and CD151 (29), and HCC cells expressing α6β4 integrin can use laminin 332 as a preferential route to invade surrounding tissues whereas those not expressing α3β1 integrin do not migrate and invade (23, 36). In addition, recent reports suggested that the α3 chain of integrin, but not α6, causes tumor cell invasion and MMP activation in HCC, and that the TGF-β1 induction of α3 induces migratory and invasive phenotypes in noninvasive HCC cell lines (48, 49). Our data showed that NDRG2 could suppress the TGF-β1-induced expression of α3β1 integrin and β3/γ2 subunits of laminin 332 in HCC cells. Moreover, in the absence of TGF-β1 treatment, the basal levels of α3 integrin and γ2 subunit of laminin 332 were also reduced by NDRG2 in PLC/PRF/5 cells (data not shown). As a result, NDRG2 blocked the activation of Rho GTPase activity, which accompanies the engagement of α6β4 integrin with laminin 332. However, NDRG2 did not alter the expression pattern of α3β1 integrin. These data strongly suggest that the specific down-regulation of laminin 332 pathway components (α3β1 integrin and laminin 332) and MMP2...
by NDRG2 collectively inhibits the ECM-based, Rho-driven tumor cell invasion and migration, eventually playing crucial roles in suppressing tumor metastasis in HCC. Our results also suggest that NDRG2 may play important roles in antagonizing the invasion-promoting activity of TGF-β1 during metastasis. We think that these results might apply to a number of additional cancer types other than HCC because NDRG2 is frequently down-regulated in many other cancer types as well, according to public gene expression databases such as CGAP7 and SOURCE.8 In fact, we have observed that NDRG2 can also suppress the metastasis formation of melanoma (B16F10) and colon cancer (SW480) cells in mouse models of tumor metastasis (data not shown).

In summary, we reported the frequent loss of NDRG2 expression in HCC caused by hypermethylation of promoter CpG sites, the significance of the relationship between NDRG2 expression loss and aggressive clinicopathologic features of HCC patients, and the involvement of NDRG2 in the inhibition of tumor cell invasion and migration. Our results provided the first evidence that NDRG2 could contribute to the negative regulation of liver cancer metastasis by itself and to TGF-β1-mediated processes. Further investigations on the molecular mechanism of NDRG2 action may offer a novel approach for treating HCC, especially targeting the tumor metastasis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
18. NDRG2 is a Novel Metastasis Suppressor of Liver Cancer...


Functional and Clinical Evidence for *NDRG2* as a Candidate Suppressor of Liver Cancer Metastasis

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