

N-myc downstream-regulated gene 1 inhibits the proliferation and invasion of hepatocellular carcinoma cells via the regulation of integrin β 3

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Abstract. N-myc downstream-regulated gene 1 (NDRG1) is a multifunctional protein associated with carcinogenesis and tumor progression. The function of NDRG1 in hepatocellular carcinoma (HCC) cells remains controversial. The present study investigated the role of NDRG1 in HCC as well as its molecular mechanism using a range of techniques, including western blot analysis, cellular proliferation test, wound healing assay and Transwell assay. In HCC, the levels of NDRG1 expression were highest in the cytoplasm, followed by the membrane, and were lowest in the nucleus. NDRG1 was revealed to inhibit the proliferation and invasion of BEL7402 cells, which facilitated the hypothesis that NDRG1 expression levels may be lower in cell line with a high metastatic potential compared with those in cell lines with a low metastatic potential. However, the present study identified that NDRG1 expression was higher in detached BEL7402 cells and MHCC-97H cells compared with that in attached BEL7402 cells and MHCC-97L cells. Thus, this finding was contrary to what was expected, suggesting that NDRG1 overexpression in the HCC with a high metastatic potential may be the compensatory mechanism. The human HCC BEL7402 cell line demonstrated a significant

increase in the capability of motility, invasion and cellular proliferation following NDRG1-short hairpin RNA transfection. Integrin β 3 (ITGB3) protein expression was increased in NDRG1-downregulated BEL7402 cells and SMMC7721 cells compared with that in the control cells. The present study suggested that NDRG1 may be a potential anti-tumor target for the treatment of patients with HCC. A potential mechanism for these roles of NDRG1 is by regulating ITGB3 expression; however, this requires additional investigation.

Introduction

Liver cancer is the fifth most frequently diagnosed type of cancer worldwide in males; however, it is the second most common cause of cancer-associated mortality. In women, liver cancer is the seventh most commonly diagnosed type of cancer and the sixth leading cause of cancer-associated mortality (1). Hepatocellular carcinoma (HCC) is the most common form of liver cancer, and accounts for ~70-85% of all cases (1). Patients with HCC typically exhibit few early symptoms, and thus, have low early diagnostic rates. Cases are usually confirmed at a late stage, which is past the most opportunistic time for surgery (2). Due to poor prognosis, metastasis and recurrence are likely to occur following surgery, the 5-year survival rate for patients with HCC is 30-40% (3). Current efforts are focused on identifying reliable biomarkers to predict HCC occurrence and development.

The N-myc downstream-regulated gene 1 (NDRG1; also termed Drg1, cap43, RTP, Rit2 and PROXY-1) belongs to the NDRG gene family. The NDRG1 gene is positioned at chromosome location 8q24.2 (4) or 8q24.3 (5). The NDRG1 gene has 60,085 bp, including 16 exons and 15 introns, and encodes a 2997-bp RNA with a 1182-bp region that translates into the NDRG1 protein (4). The NDRG1 protein is ~42,835 Da in length, and consists of 394 amino acids (6,7). In normal liver tissue, the NDRG1 protein is generally expressed in biliary epithelial cells but not in hepatocytes (8). In addition, NDRG1 protein staining is not affected by the condition of the liver (such as hepatitis or cirrhosis) or the type of infecting hepatitis virus (9).

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Abbreviations: NDRG1, N-myc downstream-regulated gene 1; HCC, hepatocellular carcinoma; ITGB3, integrin β 3; CCK-8, Cell Counting Kit-8; MMP10, matrix metalloprotease 10; SERPINE1, serine protease inhibitor clade E member 1; TNFSF10, tumor necrosis factor receptor superfamily member 10

Key words: NDRG1, ITGB3, HCC, invasion, proliferation, metastasis

In hepatoma cells, NDRG1 expression has been observed to be significantly increased compared with normal hepatocytes, and NDRG1 protein was generally expressed in the cytoplasm and membrane, but rarely in the nucleus (8-10). However, a previous *in vitro* study revealed that DNA damage increased the levels of NDRG1 nuclear expression (11). Previously, NDRG1 has been defined as an anti-tumor gene in several cancers due to its involvement in tumor invasion, metastasis and proliferation (12-16). However, theories regarding the function of NDRG1 vary across different studies. For example, a previous study stated that NDRG1 suppressed tumor growth in HCC (17). By contrast, other studies have suggested that NDRG1 exerted a stimulating effect on HCC (18,19). Therefore, the present study utilized a series of experiments in order to clarify the function of the NDRG1 gene in different HCC cell lines *in vitro*, and used a polymerase chain reaction (PCR) array test to determine downstream associated genes that may also be regulated by NDRG1. The present study identified that knockdown of NDRG1 expression resulted in the upregulation of several genes, including integrin $\beta 3$ (ITGB3).

ITGB3, also termed platelet glycoprotein III and cluster of differentiation 61, belongs to the integrin family. Within this family of cell surface receptors and adhesion molecules, ITGB3 regulates cellular proliferation, migration, cell survival and cell morphology, performs a main role in the processes of cell adhesion and movement, and can affect tumor growth and metastasis (20). However, the exact role of ITGB3 during tumor growth and metastasis requires additional studies. The integrin $\alpha \beta 3$ and $\alpha \text{IIb} \beta 3$ are members of the ITGB3 family. Integrin $\alpha \beta 3$ is mainly expressed on the surface of endothelial cells, smooth muscle cells, monocytes and platelets (21). Integrin $\alpha \beta 3$ has been observed to affect tumor angiogenesis and is also strongly expressed in malignant tumor angiogenic endothelial cells (22). Integrin $\alpha \beta 3$ could promote tumor cell growth and angiogenesis in melanoma and breast cancer (23,24) and blocking integrin $\alpha \beta 3$ resulted in reduced proliferation and invasion in ovarian cancer (25). Furthermore, integrin $\alpha \text{IIb} \beta 3$ is mainly expressed in platelets and megakaryocytes, and it has been observed to regulate platelet interacting with tumor cells, which might help to tumor metastatic spread (20). Currently, correlation studies between NDRG1 and ITGB3 have not been reported. In the present study it was shown that knockdown of NDRG1 expression resulted in the upregulation of ITGB3. However, the regulatory mechanism between NDRG1 gene expression and ITGB3 function requires additional investigation.

Materials and methods

Tissue samples. The tissue microarray (Hliv-HCC150cs-01) was purchased from Shanghai Outdo Biotech Co., Ltd. (Shanghai, China), and included 75 specimens of cancerous HCC tissue and corresponding adjacent degenerative tissue. Table I describes the patient characteristics of the study population.

Cell lines and culture. The human HCC BEL7402 and SMMC7721 cell lines, the human colon carcinoma SW480 and SW620 cell lines, a cell line with a high metastatic potential (MHCC-97H) and a cell line with a low metastatic potential

(MHCC-97L) were all obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The BEL7402, SMMC7721, SW480 and SW620 cell lines were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The MHCC-97H and MHCC-97L cell lines were maintained in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% (v/v) fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% antibiotic solution (Gibco™ Penicillin-Streptomycin; Thermo Fisher Scientific, Inc.) and incubated at 37°C in a humidified incubator under 5% CO₂. The detach BEL7402 cells mimic the metastatic cancer cells in the vascular system. Access method of attach and detach BEL7402 cells was used according to our previous description (26).

Immunohistochemistry (IHC). The IHC staining method was used according to a previous description (27).

Plasmid construction and transfection. Three candidate NDRG1 knockdown plasmids were purchased from Shanghai Genechem Co., Ltd. (Shanghai, China). The BEL7402 cells were transfected with the short hairpin (sh) RNA plasmid using the jetPRIME® transfection reagent (Polyplus-transfection, Illkirch, France) according to the manufacturer's protocol. The shRNA sequence was 5'-CTCTAAACAACCCTGAGAT-3', and was designed according to the sequence provided under the GenBank accession number NM_001258432.1 (28). The shRNA sequence was inserted into the eukaryotic expression vector GV102 (GeneChem Co., Ltd., Shanghai, China). The manufacturer's protocol was followed for the transfection, using non-transfected and untreated cells as controls. Total RNA was extracted 48 h post-transfection, and total protein was extracted 72 h post-transfection. Western blot analysis and quantitative (q) PCR were used to validate the effects of NDRG1 downregulation in transfected HCC cells.

Western blot analysis. Experiments were conducted at least three times. Total protein was extracted using a radio immunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) and separated using SDS-PAGE (12% separating gel and 5% stacking gel). A total of 20 μg of protein was loaded into each well. The target proteins were then transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Subsequent to being blocked with 5% non-fat milk for 2 h at room temperature, the membranes were incubated with a primary anti-human NDRG1 antibody (catalog no. PA5-18109; dilution, 1:1,000; Pierce, Thermo Fisher Scientific, Inc.) or anti-human ITGB3 antibody (catalog no. 13,166; dilution 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) at 4°C overnight. The membranes were then incubated with a secondary peroxidase-conjugated affiniPure rabbit anti-goat IgG antibody (cat. no. ZB2306; dilution, 1:10,000; Zhong Shan Jin Qiao Inc., Beijing, China) for NDRG1 and goat anti-rabbit IgG antibody (cat. no. ZB2301; dilution, 1:10,000; Zhong Shan Jin Qiao Inc.) for integrin $\beta 3$ at room temperature for 1 h. Rabbit anti-GAPDH (dilution, 1:1,000; Hangzhou Goodhere Biotechnology Co., Ltd., Hangzhou, China) was used as the control. An enhanced chemiluminescence method was used

to visualize western blot results (cat. no. WBLUC0100; EMD Millipore). Protein band densitometry was measured using Image J2x (National Institutes of Health, Bethesda, MA, USA).

qPCR. Experiments were conducted at least three times. BEL7402 cells transfected with empty plasmid vector (BEL7402^{vec}) and BEL7402 cells were as negative controls. Total RNA was extracted from transfected and non-transfected BEL7402 cells using the Quick-RNA extraction reagent (Ambion; Thermo Fisher Scientific, Inc., USA). qPCR was performed using the SYBR Green Premix Ex TaqTM (catalog no. RR420Q; Takara Bio, Inc., Otsu, Japan) following the manufacturer's protocol, using 35 cycles of 94°C for 30 sec and 60°C for 30 sec. The primers for NDRG1 were as follows: Forward, 5'-AACCCACACAGTCACCCTGC-3' and reverse, 5'-ACTCCACCACGGCATCCACT-3'. The primers for GAPDH were as follows: Forward, 5'-GAGAAGTATGAC AACAGCCTCAA-3' and reverse, 5'-TGAGTCCTTCCACGA TACCAA-3'. The 2^{-ΔΔC_q} method was used as the normalization method for analysis (29).

PCR array assay. The RT² ProfilerTM PCR Array Human Tumor Metastasis kit was purchased from Qiagen, Inc. (catalog no. 330231; Valencia, CA, USA). The procedures for RNA purification, complementary DNA first strand synthesis and qPCR were conducted according to the manufacturer's protocols. The primers for 84 genes known to be involved in metastasis were provided. The reaction system for qPCR was prepared according to the manufacturer's protocol, using 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Briefly, 20 μl of the total reaction mixture was added to each well of a 96-well plate containing 5 μg RNA. Data analysis was completed using the Web-based PCR Array Data Analysis Software on the Qiagen website (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>).

Cellular proliferation test. A cell suspension of 100 μl (20,000 cells) was inoculated in each well of a 96-well plate. Subsequently, cells were cultured at 37°C in a humidified incubator with 5% CO₂ overnight. Cellular proliferation was assessed using a Cell Counting Kit-8 assay (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) assay according to the manufacturer's protocol. In briefly, 10 μl of the CCK-8 assay solution was added to each well and incubated at 37°C in a humidified incubator with 5% CO₂ for 2 h. Absorbance values were then measured at 450 nm with a spectrophotometer.

Wound healing assay. A total of 1x10⁶ cells were seeded into 35-mm dishes, and when they reached 90% confluence, a scratch was created with a 200-μl pipette tip. Subsequently, cells were cultured in a serum-free RPMI-1640 medium at 37°C in a humidified incubator with 5% CO₂ for the next 48 h. Micrographs were captured at 0 and 48 h. Three separate studies were conducted, and Image-Pro Plus version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) was used for data analysis.

Transwell migration and Matrigel invasion assays. For Matrigel invasion assay, 1x10⁵ cells were seeded in the upper

Table I. Characteristics of the study population (n=75).

Categorical variables	Patients, n (%)
Gender	
Female	11 (14.7)
Male	64 (85.3)
Age, years (mean ± standard deviation)	52.5±10.5
AJCC cancer staging	
I	23 (30.7)
II	26 (34.7)
III	23 (30.7)
IV	3 (4.0)

AJCC, American Joint Committee on Cancer.

chamber with Matrigel (NO. 356,234; BD Biosciences, USA) in serum-free medium for 20 h as previously described (30). Transwell migration assay was conducted without Matrigel and cultured for 6 h as previously described (30). Subsequently, the membrane was swabbed to remove the cells in the upper chamber, and the membrane was stained with 0.5% crystal violet dissolved in methanol. Image-Pro Plus version 6.0 was used to count the number of cells that adhered to the membrane of the inserts. A total of three replicates were performed.

Statistical analysis. All data were processed using the SPSS 19.0 statistical software program (IBS SPSS, Armonk, NY, USA). Comparisons between 2 groups were performed using an independent sample t-test and comparisons among >2 groups were performed using one-way analysis of variance. Correlation analyses were conducted using the Spearman's rank correlation coefficient analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

NDRG1 protein expression in HCC tissue. The present study analyzed the characteristics of NDRG1 protein expression in HCC tissue. The NDRG1 protein was primarily expressed in the cytoplasm, membrane and nucleus of cancerous cells and the corresponding adjacent degenerative cells. The NDRG1 protein immunoreactivities differed in various subcellular locations; cytoplasm immunoreactivity was the strongest, followed by that in the membrane, and was weakest within the nucleus. No significant difference was identified between NDRG1 expression in the cytoplasm or nucleus of cancerous cells compared with that of the corresponding adjacent degenerative cells. However, membrane expression in cancer cells was significantly higher compared with the expression of NDRG1 in adjacent degenerative cells (P<0.05; Fig. 1A-C). There was also a negative correlation between the NDRG1 cytoplasm staining in cancerous cells and tumor-node-metastasis (TNM) stage (P<0.01; r_s=-0.333; Fig. 1D) (31). In other words, increased NDRG1 cytoplasm expression was observed in tumors with earlier TNM stages compared with tumors with later TNM stages. A positive correlation was observed between

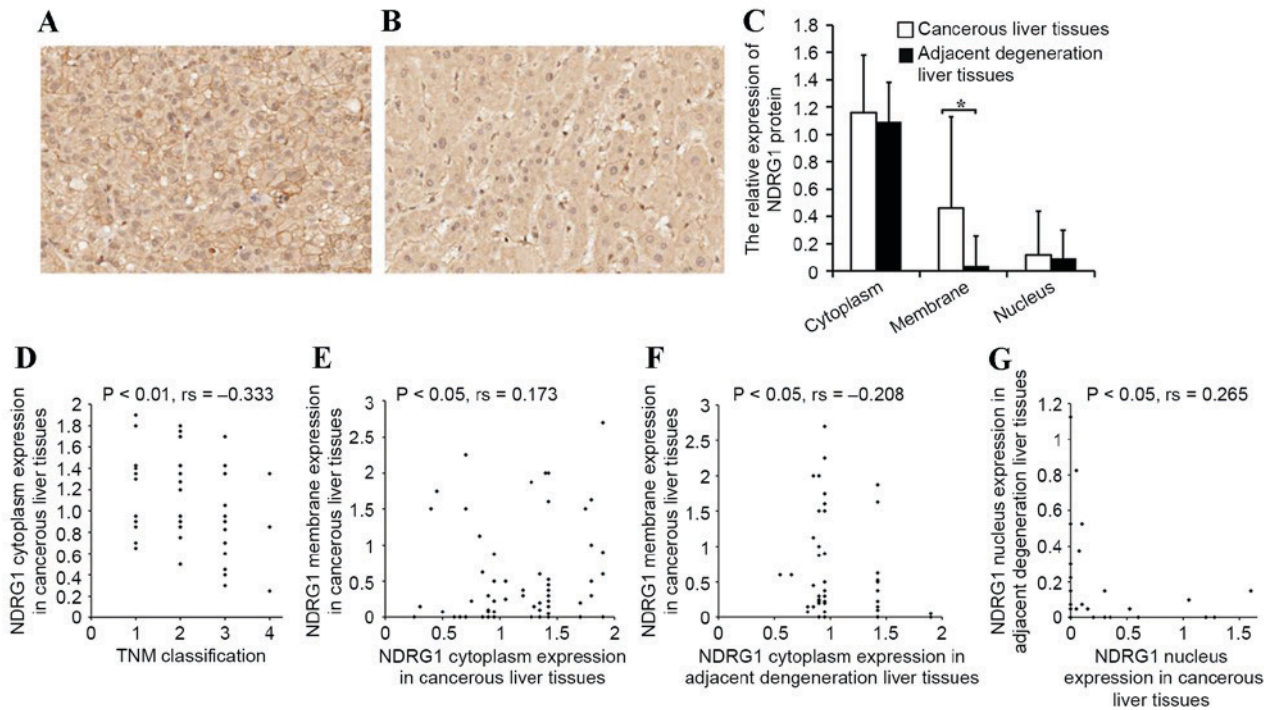


Figure 1. Immunohistochemistry results of NDRG1 protein in human hepatocellular cancer cells and adjacent degenerative cells, and results of Spearman's correlation tests between NDRG1 protein expression and clinical parameters. (A) NDRG1 protein expression in cancerous cells. (B) NDRG1 protein expression in adjacent degenerative cells. (C) Analysis of NDRG1 protein expression in the cytoplasm, membrane and nucleus of cancerous and adjacent degenerative cells. * $P < 0.05$. (D) Negative correlation between NDRG1 cytoplasm expression and TNM classification ($P < 0.01$; $r_s = -0.333$). (E) Positive correlation between NDRG1 cytoplasm expression and membrane expression ($P < 0.05$; $r_s = 0.173$). (F) Negative correlation between NDRG1 membrane expression in cancerous cells and NDRG1 cytoplasm expression in adjacent degenerative cells ($P < 0.05$; $r_s = -0.208$). (G) Positive correlation between NDRG1 nucleus expression in adjacent degenerative cells and cancer cells ($P < 0.05$; $r_s = 0.265$). The scale bar in (A and B) was 4.167 pixels/ μm and the magnification used for the microscope was 10x20. TNM, tumor-node-metastasis; NDRG1, N-myc downstream-regulated gene 1.

NDRG1 membrane expression and cytoplasmic expression in cancer cells ($P < 0.05$; $r_s = 0.173$; Fig. 1E). A negative correlation was also identified between membrane expression in cancerous cells and cytoplasmic expression in adjacent degenerative cells ($P < 0.05$; $r_s = -0.208$; Fig. 1F). A positive correlation was also observed between NDRG1 nucleus expression in adjacent degenerative cells and cancer cells ($P < 0.05$; $r_s = 0.265$; Fig. 1G).

NDRG1 expression levels in different cell culture models. The present study analyzed the expression of NDRG1 mRNA in different cell culture models using qPCR. The results revealed an increase in NDRG1 messenger (m)RNA expression in detached human HCC BEL7402 cells compared with that in attached BEL7402 cells (Fig. 2A). The NDRG1 mRNA expression levels were also higher in MHCC-97H cells compared with those in MHCC-97L cells ($P < 0.05$; Fig. 2B). The NDRG1 mRNA expression levels in colon carcinoma SW620 cells were lower when compared with those in colon carcinoma SW480 cells ($P < 0.05$; Fig. 2C).

NDRG1 expression is significantly downregulated in NDRG1-shRNA-transfected BEL7402 cells. To investigate NDRG1 expression following the knockdown of the NDRG1 gene with shRNA, the present study performed a western blot analysis and qPCR on transfected and control cells. The western blot analysis and qPCR demonstrated that NDRG1 expression was significantly decreased in the BEL7402 cells

transfected with NDRG1-shRNA compared with that in the control cells ($P < 0.05$; Fig. 3A-C).

NDRG1 inhibits invasion and metastasis in BEL7402 cells. Transwell migration assay demonstrated that NDRG1-shRNA BEL7402 cells were significantly more capable of motility compared with BEL7402 cells transfected with empty plasmid vector ($P < 0.05$; Fig. 4A and B). The Matrigel invasion assay revealed that the BEL7402 cells had a significantly higher invasive ability with NDRG1 knockdown ($P < 0.05$), indicating that NDRG1 may inhibit HCC cell invasion *in vitro* (Fig. 4C and D). The wound healing assay also showed that NDRG1-shRNA BEL7402 cells were significantly more capable of movement compared with BEL7402 cells transfected with empty plasmid vector ($P < 0.05$; Fig. 4E and F). Therefore, NDRG1 had the ability to inhibit HCC cell migration and invasion *in vitro*.

NDRG1 inhibits BEL7402 cellular proliferation. The CCK-8 assay was performed to detect the proliferation of BEL7402 cells at a 48 h following transfection with NDRG1-shRNA. The results demonstrated that BEL7402 cellular proliferation was enhanced due to NDRG1 knockdown. Therefore, NDRG1 may inhibit BEL7402 cellular proliferation at a level that was statistically significant ($P < 0.05$; Fig. 5).

Downstream molecular candidates of NDRG1 inhibit tumor metastasis. The PCR array assay was used to determine

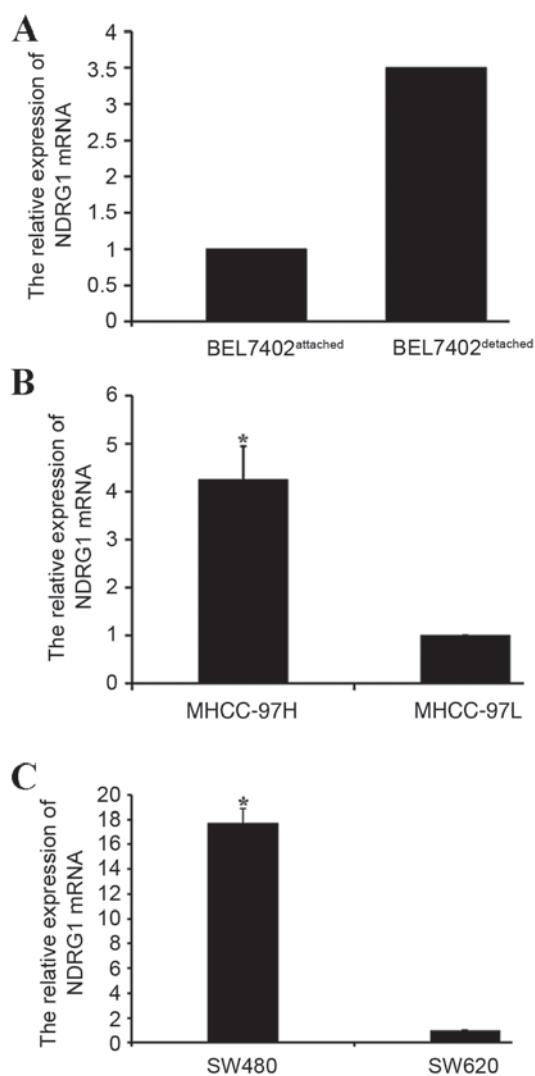


Figure 2. NDRG1 expression of different cell culture models detected by quantitative polymerase chain reaction. (A) NDRG1 mRNA expression in detached and attached BEL7402 cells. (B) NDRG1 mRNA expression in MHCC-97H and MHCC-97L cells. (C) NDRG1 mRNA expression in SW620 and SW480 cells. * $P < 0.05$. NDRG1, N-myc downstream-regulated gene 1; mRNA, messenger RNA; MHCC-97H, a cell line with a high metastatic potential; MHCC-97L, a cell line with a low metastatic potential; HCC, hepatocellular carcinoma.

the downstream gene candidates of NDRG1 that inhibited tumor metastasis. The results demonstrated that NDRG1 knockdown upregulated ITGB3, MMP10 and SERPINE1 and downregulated TNFSF10 in both BEL7402 and SMMC7721 cell lines (Fig. 6A). Furthermore, western blot analysis was used to determine ITGB3 protein expression. The results demonstrated that ITGB3 protein expression was increased in NDRG1-downregulated BEL7402 and SMMC7721 cells compared with that in the control cells, indicating that the NDRG1 gene may suppress tumor metastasis by regulating ITGB3 expression ($P < 0.05$; Fig. 6B and C).

Discussion

Currently, the function of the NDRG1 gene is controversial (17-19); therefore, the present study investigated the role of NDRG1 in HCC as well as its molecular mechanism. Firstly,

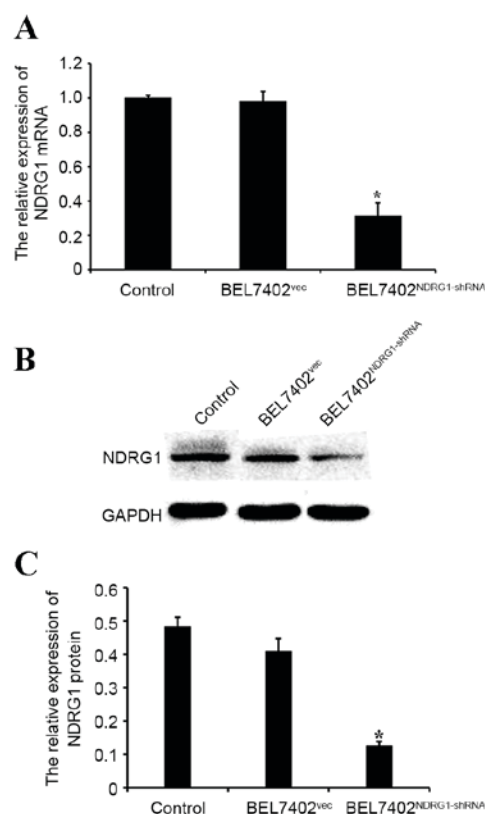


Figure 3. NDRG1-shRNA interfering efficiency in BEL7402 cells. (A) Quantitative polymerase chain reaction detected NDRG1-shRNA interfering efficiency in BEL7402 cells. (B) Western blot analysis of NDRG1 protein expression levels in BEL7402^{vec}, BEL7402^{NDRG1-shRNA} and control cells. (C) NDRG1 protein expression histogram in BEL7402^{vec}, BEL7402^{NDRG1-shRNA} and control cells. * $P < 0.05$ vs. control. NDRG1, N-myc downstream-regulated gene 1; vec, vector; shRNA, short hairpin RNA.

NDRG1 expression was detected in HCC tissue and cells. The present results demonstrated that NDRG1 was expressed in cancerous liver cells and adjacent degenerative liver cells. A previous study observed that NDRG1 was expressed in 6% of cirrhosis and benign liver lesions subsequent to staining (10). These results indicated that NDRG1 may participate in the full progression of the occurrence and development of HCC, from liver cell degeneration to malignant changes. Although NDRG1 stained stronger on the membrane of liver cancerous cells compared with that in the adjacent degenerative liver cells, there was no staining difference in the cytoplasm or nucleus. As mentioned previously, the expression of the NDRG1 gene was always negative in normal liver cells (8-10,18). Therefore, the levels of NDRG1 expression in liver cell membrane may estimate the degree of injury in cells or the extent of cell cancerization.

A previous study demonstrated that high NDRG1 expression in patients with HCC was associated with a short overall survival rate and a poor prognosis. High NDRG1 expression was detected in poorly differentiated HCC and high TNM stages (10). However, when the present study considered NDRG1 expression in different subcellular localizations, it identified that there was a negative correlation between the cytoplasmic expression of NDRG1 in cancerous liver cells and the TNM stage, which indicates that higher expression levels were observed in smaller sized tumors. In our previous study,

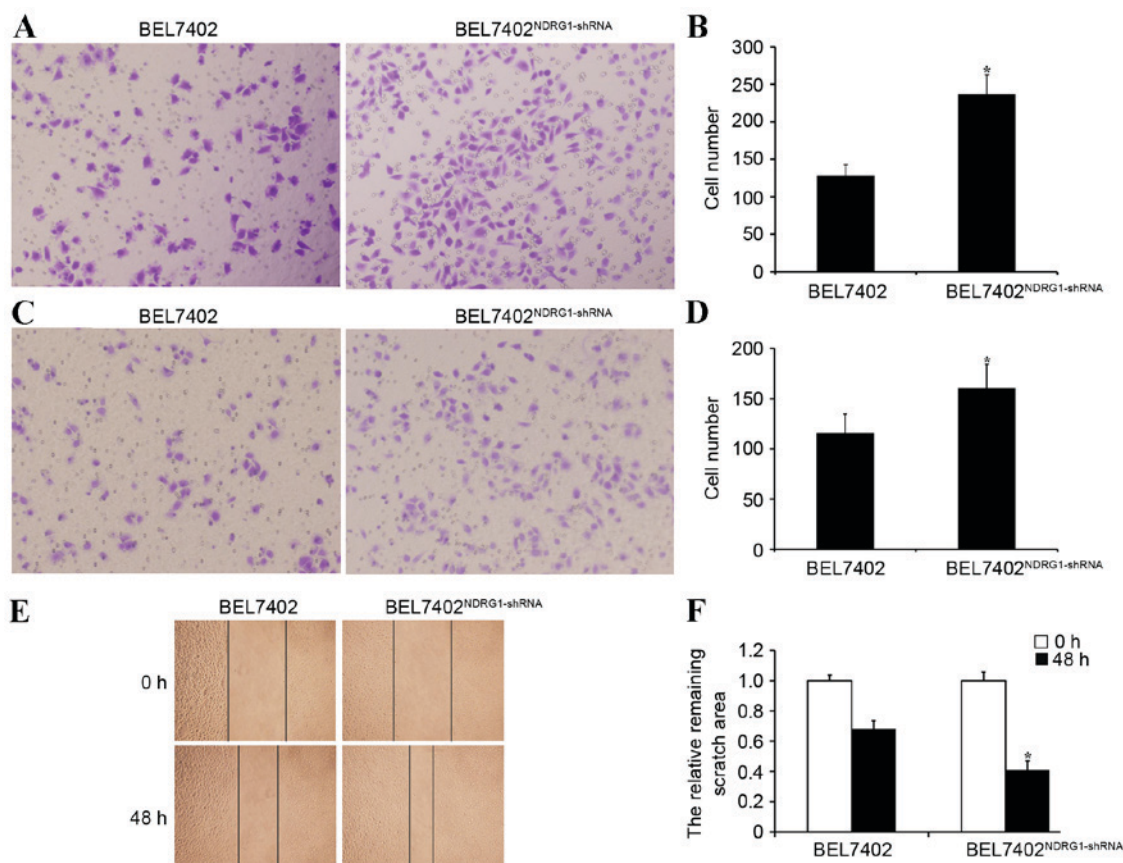


Figure 4. NDRG1 inhibits the invasion, metastasis and proliferation abilities of BEL7402 cells. (A) Transwell migration assay results demonstrated the invasion ability of BEL7402 cells following NDRG1 knockdown. (B) Quantification of the Transwell migration assay demonstrated that BEL7402 cells had significantly increased invasion ability following NDRG1 knockdown ($P < 0.05$). (C) Matrigel invasion assay showing the differences in motility of BEL7402 cells following NDRG1 knockdown. (D) Quantification of the Matrigel invasion assay demonstrated that BEL7402 cells had significantly increased motility following NDRG1 knockdown ($P < 0.05$). (E) Wound healing assay demonstrating the difference in cell movement prior to and subsequent to NDRG1 downregulation. (F) Results of the wound healing assay after 0 and 48 h demonstrated increased cell movement capability subsequent to NDRG1 downregulation ($P < 0.05$). $^*P < 0.05$. The scale bar in (A and C) is $4.167 \text{ pixels}/\mu\text{m}$ and in (E) is $2.083 \text{ pixels}/\mu\text{m}$. The magnification used in (A and C) was 10×20 and for (E) was 10×10 . NDRG1, N-myc downstream-regulated gene 1; shRNA, short hairpin RNA.

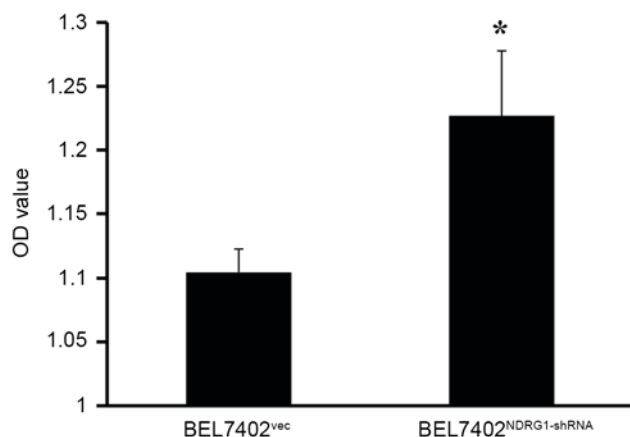


Figure 5. Cell Counting Kit-8 assay demonstrated that the proliferation of BEL7402 cells was significantly enhanced following NDRG1 knockdown ($P < 0.05$). $^*P < 0.05$. OD, optical density; NDRG1, N-myc downstream-regulated gene 1; vec, vector; shRNA, short hairpin RNA.

it was revealed that the cytoplasmic expression of NDRG1 may be associated with lymph node metastasis (27).

The expression of NDRG1 may therefore become a marker of the degree of invasiveness for local tumors, although the

NDRG1 expression observed in the nucleus of the cancerous and degenerative liver cells in the present study may be coincidental. However, there was a positive correlation between the expression of NDRG1 in the nucleus of cancerous liver cells and that in the adjacent degenerative liver cells, indicating that the regulating factor of NDRG1 nuclear expression acts equally on cancerous and degenerative liver cells. However, WoLF PSORT (an advanced protein subcellular localization prediction tool) and amino acid sequence analysis demonstrated that the NDRG1 protein lacked the motifs used for localization in nucleus (32). Thus, NDRG1 nucleus localization may rely on protein phosphorylation. Several phosphorylation sites have been identified in the C-terminal site of the NDRG1 protein (33).

Reports have shown that serum- and glucocorticoid-induced protein kinase 1 could phosphorylate NDRG1 on the amino acids Thr328, Ser330, Thr346, Thr356 and Thr366 (34,35). Glycogen synthase kinase 3β (GSK3 β) was shown to be able to phosphorylate Ser342, Ser352 and Ser362 of NDRG1 (36). These results are valuable to understanding the role of NDRG1, since protein phosphorylation is reversible and affects NDRG1 subcellular localization in the cell (37). In addition, NDRG1 can localize in the nucleus subsequent to binding to the 70-kDa heat shock cognate protein (Hsc70) (38). Hsc70 is a molecular

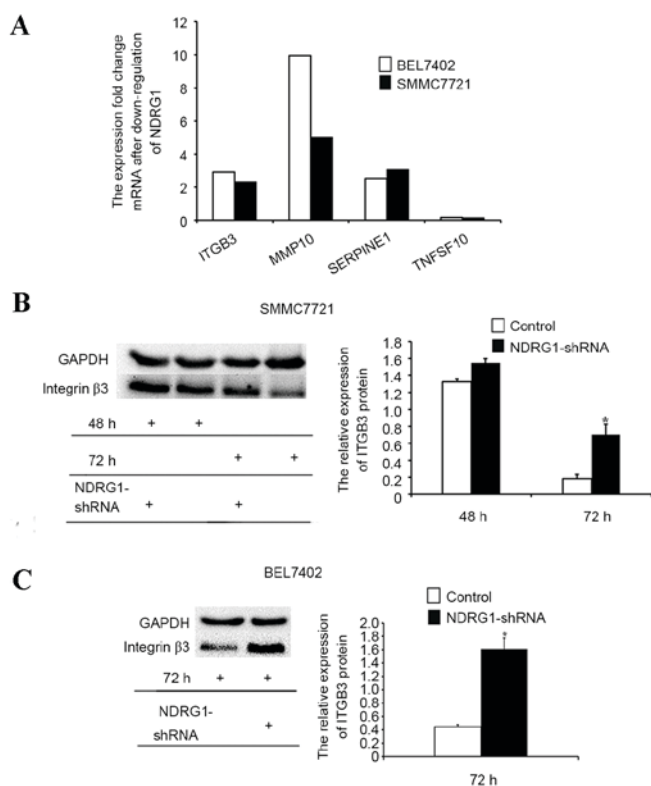


Figure 6. Results of polymerase chain reaction demonstrating that the down-regulation of NDRG1 in BEL7402 and SMMC7721 cell lines resulted in subsequent up- or downregulation of different genes. (A) Effect of NDRG1 on the expression levels of ITGB3, MMP10, SERPINE1 and TNFSF10 in the BEL7402 and SMMC7721 cell lines. ITGB3 protein expression was detected by western blot analysis prior to and subsequent to the downregulation of the NDRG1 gene in (B) BEL7402 and (C) SMMC7721 cells. * $P < 0.05$ vs. control. NDRG1, N-myc downstream-regulated gene 1; ITGB3, integrin β 3; MMP, matrix metalloprotease; SERPINE1, serine protease inhibitor clade E member 1; TNFSF10, tumor necrosis factor receptor superfamily member 10; shRNA, short hairpin RNA.

chaperone that mediates mast cell transport between the cytoplasm and nucleus (39).

NDRG1 was revealed to inhibit the proliferation and invasion of BEL7402 cells, which facilitated the hypothesis that NDRG1 expression levels may be lower in a cell line with a high metastatic potential compared with those in a cell line with a low metastatic potential. However, higher expression of NDRG1 mRNA was detected in MHCC-97H cells compared with that in MHCC-97L cells. In addition, higher expression of NDRG1 mRNA was detected in detached BEL7402 cells compared with that in attached BEL7402 cells. Thus, these findings were contrary to the expected results, and suggest that NDRG1 overexpression may be the compensatory mechanism. To clarify the expression trend of different metastatic potential cells lines, the present study detected the NDRG1 mRNA expression of colon carcinoma SW480 and SW620 cells. SW480 cells and SW620 cells were respectively derived from primary tumor and metastatic lymph nodes of the same person, which may make clear the expression of NDRG1 in primary and metastatic tissues. The result showed that NDRG1 expression was lower in SW620 compared with SW480 cells, which was contrary to the results of HCC cell lines. Maybe the NDRG1 expression was tissue specific. It has been reported

that the level of RNA polymerase II bound to the NDRG1 promoter was lower in SW620 cells compared with SW480 cells, which reduced histone H4 acetylation, and enhanced histone H3 Ser10 phosphorylation (40). The unique histone modifications may be the possible mechanism for the different expression of NDRG1 in cell lines of different metastatic potential (40).

A previous study identified that NDRG1 inhibited the proliferation and invasion of cancer cells. In prostate cancer cells, the aberrant methylation of NDRG1 CpG islands caused downregulation of the NDRG1 promoter, leading to accelerated cellular proliferation and invasion of cancer cells (41). To illustrate the function of NDRG1 in HCC, the present study performed a series of *in vitro* experiments. Knockdown of the NDRG1 gene increased the proliferation of BEL7402 cells, indicating that NDRG1 inhibits the proliferation of hepatoma cells *in vitro*, which is consistent with the results of a previous study (17). However, a study in Hep3B and HepG2 cells revealed that the downregulation of NDRG1 decreased cell growth (19). A previous study indicated that NDRG1 directly interacted with GSK3 β and Nur77 (also known as NR4A1, nuclear receptor subfamily 4 group A member 1) (42). This interaction could prevent the degradation of β -catenin, and consequently regulate β -catenin-relevant downstream signaling pathways to promote the growth of Hep3B and HepG2 cells (42). A previous study demonstrated that NDRG1 induced G₀/G₁-phase cell cycle arrest in HCC cell lines, and may incorporate cell cycle regulators such as p21 and cyclin-dependent kinase 4 in the NDRG1-induced cell cycle arrest (17). The suppression of NDRG1 inhibited tumor growth by inducing extensive cellular senescence in HCC cells (17,43).

To confirm the controversial function of NDRG1 in the metastasis of HCC, NDRG1 was knocked down using NDRG1-shRNA in BEL7402 cells in the present study, demonstrating that NDRG1 downregulation promotes HCC cell invasion and metastasis. This result indicates that NDRG1 inhibits the invasion and metastasis of HCC cells. A previous study demonstrated that NDRG1 overexpression maintained membrane E-cadherin and β -catenin levels while inhibiting transforming growth factor (TGF)- β -stimulated cellular migration and invasion (44), and this finding indirectly confirmed the results of the present study. A previous study demonstrated a potential mechanism by which NDRG1 effectively inhibited rat prostate cancer AT6.1 cells from metastasizing to the lungs (45). This mechanism may also regulate the cell structural protein actin. Therefore, NDRG1 could affect the formation and regulation of actin filaments by inhibiting the rho-associated protein kinase 1/phosphorylated myosin light chain 2 signaling pathway (46). A recent study has also demonstrated that NDRG1 could suppress tumor metastasis by inhibiting the focal adhesion kinase/paxillin signaling pathway (47).

To investigate the mechanism by which NDRG1 is involved in the proliferation and metastasis of HCC, the present study screened the downstream gene candidates regulated by NDRG1 that inhibited tumor metastasis. The results demonstrated that, in the BEL7402 and SMMC7721 cell lines, a decrease in NDRG1 expression led to an increase in ITGB3 expression. The expression levels of the ITGB3 protein were detected using western blot analysis, and were consistent with the PCR array

results. A previous study has demonstrated that integrin $\alpha\beta3$ could increase adhesion and invasion of HCC (48). ITGB3 could enhance the TGF- $\beta1$ /H₂O₂/HOCl-inducing invasive ability of non-metastatic HCC cells via the TGF- $\beta1$ signaling pathway (49).

In addition, reducing the levels of integrin $\alpha\beta3$ suppressed the cathepsin B-induced proliferation of HCC (50). These previous studies confirmed the findings of the present study. Integrin signaling induced cytoskeleton organization change and contraction, which consequently promoted cellular migration. A potential mechanism by which this happens could be that activated integrin $\alpha\beta3$ was not effective for the primary tumor growth, but it could collaborate with platelets in order to promote tumor cell metastasis from the bloodstream (51). To the best of our knowledge, the association between NDRG1 and ITGB3 has not been studied to date. The role of NDRG1 on the inhibition of tumor metastasis and cellular proliferation was considered to occur via the regulation of ITGB3. The microarray results from colon cancer tissue demonstrated a negative correlation between NDRG1 expression in the nucleus of cancerous cells and ITGB3 stromal expression in adjacent degenerative cells. A negative correlation was also revealed between NDRG1 expression in the nucleus of cancerous cells and ITGB3 expression in cancer tissue. These results suggest that NDRG1 is involved in regulating ITGB3 expression at the transcriptional level (data not shown).

In conclusion, as a key signaling molecule regulating tumor proliferation and metastasis, NDRG1 is likely to become the target of anti-tumor metastasis or to be a biomarker observing the prognosis and metastasis of patients with tumors. In order to be considered as a drug target, additional studies are required on NDRG1 in order to identify the molecular mechanism behind its anti-tumor activity. The present study identified that NDRG1 inhibited the proliferation and metastasis of HCC by regulating ITGB3 expression at the transcriptional level. However, the association between NDRG1 and ITGB3 requires additional investigation through *in vitro* and *in vivo* experiments.

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