N-myc downstream regulated gene 1 acts as a tumor suppressor in ovarian cancer

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Abstract. Although implicated in a number of tumor types, the role of N-myc downstream regulated gene 1 (NDRG1) in ovarian cancer (OC) is unclear. In the present study, we used short hairpin RNA (shRNA) to silence NDRG1 in the OC cell line OVCAR3 and assessed the effect of its knockdown on cell morphology, proliferation, colony formation, migration and invasion. To complement these knockdown studies, we overexpressed NDRG1 in the same cell line. We found that NDRG1 knockdown significantly enhanced OVCAR3 proliferation, migration and invasion; however, there were no apparent changes in cell morphology. We also examined the effect in vivo and found that NDRG1 depletion promoted OVCAR3 xenograft growth in nude mice. In accordance with these data, we found that NDRG1 overexpression decreased proliferation, adhesion and apoptosis, and induced G0/G1 cell cycle arrest in OVCAR3 cells; expression of p21 and p53 was also increased. In conclusion, we demonstrated that NDRG1 acts as a tumor suppressor in ovarian carcinogenesis and may be a potential therapeutic target in this disease.

Introduction

Ovarian cancer (OC) arises from malignant transformation of the female ovaries. In the US in 2009, there were an estimated 21,880 newly diagnosed cases and 13,850 deaths resulting from the disease (1). The high rate of death may be attributed to late-stage diagnosis; approximately two-thirds of patients are diagnosed with stage III or IV disease (2). OC is the leading cause of death among gynecological cancers with an overall 5-year survival rate of ~19-39% (3). Unfortunately, current treatment strategies are lacking. Thus, a more comprehensive understanding of OC is required to develop additional potentially targeted therapies that can increase the survival of those diagnosed with the disease.

A number of studies have examined the genetic alterations and signal transduction changes that occur upon initiation and progression of OC (4). Despite these findings, there is still much knowledge concerning the underlying mechanisms governing OC growth that is unknown.

In the present study, we examined a possible role of N-myc downstream regulated gene 1 (NDRG1) in OC. NDRG1 belongs to a family of genes consisting of four members which all share ~57-65% sequence identity (5). NDRG1 is predominantly cytoplasmic, and the protein is ubiquitously expressed both in normal and neoplastic tissues. Additionally, it is highly conserved among multicellular organisms (6). NDRG1 has been implicated in carcinogenesis, particularly in invasion and metastasis (7,8). However, it may play distinct roles in different types of tumor. NDRG1 has been reported to act as a tumor suppressor in a number of cancers, including breast (9), prostate (10-12), pancreatic (7,8,13), cervical (14), highly metastatic colon (15) and gastric cancer (16). However, in hepatocellular carcinoma, NDRG1 may actually promote growth (17,18). The exact role of NDRG1 in OC has not been well studied. Thus, in the present study, we examined the role of NDRG1 in four OC cell lines. We both knocked down and overexpressed NDRG1 in the OC cell line OVCAR3 and found that it exhibited a growth suppressive function in this cell line. These studies provide evidence for additional, more comprehensive, analyses of NDRG1 in OC.

Materials and methods

Reagents and cell culture. Human OC cell lines HO8910, OVCAR3, SKOV3 and A2780 were obtained from the Chinese Academy of Medical Sciences. HO8910 and SKOV3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Thermo Scientific, Waltham, MA, USA). OVCAR3 and A2780 cells were cultured in RPMI-1640 medium (HyClone). All medium was supplemented with 10% fetal bovine serum (FBS) (HyClone) and 1% penicillin/streptomycin. Cells were maintained in a 37°C incubator supplied with 5% CO₂.
Table I. Short hairpin RNA sequences designed with the GenScript siRNA target finder.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>F1</td>
<td>F: 5'-gatcGGCTGGAACCTTTGTCGGAATTCAAGAGATTCGCCGACAAAGGGTTTCACGTTTTTGG-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-aattcAAAAACGTGAAACTTTTGTCGGAATTCAAGAGATTCGCCGACAAAGGGTTTCACGTTTTTGG-3'</td>
</tr>
<tr>
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<tr>
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<td>R: 5'-aattcAAAAACGTGAAACTTTTGTCGGAATTCAAGAGATTCGCCGACAAAGGGTTTCACGTTTTTGG-3'</td>
</tr>
<tr>
<td>F3</td>
<td>F: 5'-gatccGCCACAAAAACCTGCTAACATCTGAAAGAGATTCGCCGACAAAGGGTTTCACGTTTTTGG-3'</td>
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<td></td>
<td>R: 5'-aattcAAAAACGTGAAACTTTTGTCGGAATTCAAGAGATTCGCCGACAAAGGGTTTCACGTTTTTGG-3'</td>
</tr>
<tr>
<td>Control</td>
<td>F: 5'-GATCCGAGCGTTGACTGGATTTGGTCAAGAGAAGACAATCGACTGTAAGTACGAGTTTTTGG-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-AGCTTCTGACGAGTTGACTGCGATTGTCTCTTGAACAATCGGAGTTTTTGG-3'</td>
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F1, F2, F3, three different RNAi sequences targeting NDRG1; control, non-targeting sequence. siRNA, short hairpin RNA; F, forward; R, reverse; NDRG1, N-myc downstream regulated gene 1.

Construction of short hairpin RNA (shRNA) plasmids. shRNA directed against human NDRG1 was purchased from GenePharma Biotechnology (Shanghai, China); all sequences are listed in Table I. The shRNA effective target sequence for NDRG1 was 5'-TTCAAGAGA-3'. OVCAR3 cells were stably transfected with recombinant NDRG1-targeted shRNA plasmids or a control shRNA vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Stable clones were confirmed by real-time RT-PCR and western blotting. The resulting cell lines were designated as OVCAR3-shNDRG1 and OVCAR3-shNC (negative control).

Quantitative real-time PCR. Total RNA was extracted with TRIzol reagent (Invitrogen). A cDNA synthesis reaction (20 µl) was set up using PrimeScript RT reagent kit (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's instructions. Reaction conditions were as follows: 25°C for 10 min, 42°C for 30 min and 85°C for 5 min. cDNA was then amplified and detected using SYBR Premix Ex Taq™ Perfect Real-Time PCR Master Mix kit (TaKaRa). Real-time PCR was performed as follows: stage 1, pre-denaturation at 95°C for 10 min; stage 2, 40 cycles of 95°C for 15 sec, 58°C for 20 sec and 72°C for 27 sec; dissociation, 95°C for 15 sec, 60°C for 30 sec and 95°C for 15 sec. The melting curve for each primer pair was analyzed to verify specificity of the amplified product. Gene expression was determined as previously described (19). The expression levels were normalized to β-actin and relative graphs were plotted. Each experiment was performed in triplicate in 96-well plates and incubated for 24 h to allow cells to attach. After 48 h, the MTT assay was performed using 20 µl of serum-free medium containing MTT (0.5 g/l) and incubating at 37°C for 4 h. Next, 150 µl DMSO was added to each well, and all plates were shaken at room temperature for 10 min. Optical density (OD) was measured at 490 nm using a spectrophotometer microplate reader. Cellular proliferation graphs were plotted. Each experiment was performed in triplicate wells and was repeated at least three times.

Cell proliferation assay. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay kit (Beyotime, China) was used. Briefly, OVCAR3 cells transfected with shRNA-NDRG1 or shRNA-NC were plated in triplicate in 96-well plates and incubated for 24 h to allow cells to attach. After 48 h, the MTT assay was performed using 20 µl of serum-free medium containing MTT (0.5 g/l) and incubating at 37°C for 4 h. Next, 150 µl DMSO was added to each well, and all plates were shaken at room temperature for 10 min. Optical density (OD) was measured at 490 nm using a spectrophotometer microplate reader. Cellular proliferation graphs were plotted. Each experiment was performed in triplicate wells and was repeated at least three times.

Colony formation assay. Briefly, OVCAR3-shNDRG1 and OVCAR3-shNC cells were plated in 6-well plates at a density of 1,000 cells/well. After 10 days of incubation in 10% FBS containing 200 µg/ml G418, the cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet. Colonies were counted under a light microscope.

Cell migration and invasion assays. Invasion assays were performed using the BD BioCoat™ Matrigel chamber in 24-well plates (BD, USA). Resuspension solution (100 µl) containing 1.5x10^5 cells in DMEM with 1% FBS was added to the upper chamber, and 600 µl DMEM supplemented with 1% FBS was added to the lower chamber. This was followed by overnight incubation with the appropriate antibody (rabbit anti-NDRG1 monoclonal antibody, cat. no. ab32072, 1:10,000; Abcam, UK; mouse anti-β-actin monoclonal antibody, cat no. 600008-1-Ig, 1:1,000; ProteinTech, USA) at 4°C. The protein signal was detected with an ECL system (Millipore) and photographed with FluorChem E image system (Cell Biosciences).

Morphological analysis of the cultured cells. NDRG1-depleted cells (OVCAR3-shNDRG1) and control cells (OVCAR3-shNC) were seeded onto chamber slides and left overnight. The next day they were fixed with 4% paraformaldehyde, stained with 1% crystal violet and examined by light microscopy.

Western blotting. Total cell lysates were prepared with RIPA buffer containing phenylmethylsulfonyl fluoride (PMSF; Beyotime Institute of Biotechnology, China). Equal amounts of protein were separated by SDS-PAGE (10% polyacrylamide gels) and transferred to nitrocellulose membranes. The membranes were blocked with 5% milk in Tris-buffered saline containing 0.05% (v/v) Tween-20 for 1 h at room temperature.

This was followed by overnight incubation with the appropriate antibody (rabbit anti-NDRG1 monoclonal antibody, cat. no. ab32072, 1:10,000; Abcam, UK; mouse anti-β-actin monoclonal antibody, cat no. 600008-1-Ig, 1:1,000; ProteinTech, USA) at 4°C. The protein signal was detected with an ECL system (Millipore) and photographed with FluorChem E image system (Cell Biosciences).
20% FBS was added to the bottom chamber. After a 48-h incubation at 37°C in a 5% CO₂ incubator, cells in the upper well were wiped off using a cotton swab. Cells in the lower chamber were fixed, stained with H&E, and counted under a light microscope. The migration assay was performed in a similar manner except that the chambers were covered without Matrigel.

**Wound healing assay.** Cells from each group (OVCAR3-shNDRG1 and OVCAR3-shNC) were seeded into 6-well plates (5x10⁶ cells/well). The confluent monolayer was starved overnight, and then a single, linear scratch was created using a 20 µl pipette tip. After wounding, the cells were washed gently with PBS to remove cell debris and placed in fresh DMEM supplemented with 0.1% FBS to block cell proliferation. Images were captured using a phase contrast microscope at x200 magnification at 0, 24 and 48 h. The wound size was measured and analyzed using ImagineJ software. Wound closure was expressed as a percentage of the wound area at 0 h.

**Xenograft experiments.** Twenty nude mice (Balb/c athymic nude mice), aged 4-6 weeks (weighing ~20 g) were randomly divided into two groups. OVCAR3 cells were suspended in sterile PBS at a concentration of 5x10⁶ cells/ml, and 100 µl was subcutaneously injected into one flank of each mouse. Measurements were recorded every week, and changes in the average tumor volume were noted. After 7 weeks, at which time the average tumor volume reached 200 mm³ in each group, the mice were given intratumoral injections of 5 µg shNDRG1 or 5 µg shNC in 30 µl PBS every two days. Growth curves were plotted using average tumor volume of each experimental group at the set time points. The tumor size was measured with calipers in two directions, and the tumor volume (V) was calculated using the formula: \( V = (\text{length} \times \text{width})^2 \times 0.5 \).

**Construction of the NDRG1 mammalian expression vector pcDNA3.1(+)/NDRG1 and the effect of transient transfection on cell proliferation and adhesion.** The NDRG1 open reading frame was PCR amplified from OVCAR3 cells. The primer sequences used were: forward primer (NDRG1) 5’CGAAGCTTATGTCTCGGGAGATGCAG3’ and reverse primer 5’ATCTCGAGCTAGCAGGAGACCTCCAT3’. Real-time PCR was performed using the following primers: forward primer (NDRG1) 5’CGAGCTAGCAGGAGACCTCCAT3’ and reverse primer 5’ATCTCGAGCTAGCAGGAGACCTCCAT3’. Real-time PCR was performed using the following parameters: 98°C for 30 sec, followed by 35 cycles of 98°C for 10 sec, 68°C for 30 sec and 72°C for 90 sec. The final primer extension at 72°C was performed for 10 min. The PCR product was then cloned into pcDNA3.1(+) (Invitrogen) using standard techniques. Either the obtained NDRG1 expression vector pcDNA3.1(+)NDRG1 or empty vector pcDNA3.1(+) was transiently transfected into OVCAR3 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. For the cell proliferation assay, cells were seeded into a 96-well plate (2x10⁴ cells/well), and positive colonies were selected with G418 (Invitrogen) supplemented with growth medium. For the adhesion assay, wells of the 96-well culture plate were coated with Matrigel (BD Biosciences) at a concentration of 5 µg/well. Cells in medium containing 0.1% FBS were added to the wells (2x10⁵/well) and incubated at 37°C for 2 h. The number of attached cells was reported as a percentage of total cells at the end of the incubation period. Each experiment was performed in triplicate wells and repeated three times.

**Flow cytometry for cell proliferation.** Cells were transfected with control or NDRG1 expression vectors. They were then trypsinized, centrifuged at 1,000 rpm, and resuspended in 0.5 ml PBS (1X). To fix the cells, 0.5 ml 100% cold ethanol was added to each sample and incubated for 20 min. After centrifugation at 1,000 rpm for 5 min, ethanol was decanted. According to the manufacturer’s instructions, the cells were incubated in the dark in 0.5 ml propidium iodide (PI) with RNase A for 30 min at 4°C. Results were analyzed by flow cytometry. Experiments were repeated three times.

**Apoptosis assay.** Annexin V-FITC apoptosis detection kit (Abcam) was used according to the manufacturer’s instructions. Approximately 5x10⁶ cells were collected, washed twice with cold PBS, and resuspended in 500 µl of 1X binding buffer. Both 5 µl Annexin V-FITC and 5 µl PI were added, and samples were incubated in the dark at room temperature for 15 min. Apoptosis was evaluated by flow cytometry (LSR II; Becton-Dickinson, USA). The percentage of apoptotic cells was calculated using software FCS 3.0.

**Statistical analysis.** Statistical analysis was carried out using SPSS 16.0 for Windows. The two-tailed Student’s t-test was used for analyzing continuous variables. P<0.05 was considered to indicate a statistically significant result.

**Results**

**NDRG1 is expressed in the OC cell lines.** We examined NDRG1 expression by quantitative real-time RT-PCR and western blot analysis in four well-established OC cell lines. NDRG1 mRNA and protein were expressed at relatively high levels in both the OVCAR3 and HO-8910 cells; they were significantly lower in the SKOV3 and A2780 cells (Fig. 1A). We selected OVCAR3 cells for subsequent knockdown experiments as this line expressed the highest levels of endogenous NDRG1 in the four cell lines examined.

**Depletion of NDRG1 in OVCAR3 cells enhances proliferation.** Three shRNA-NDRG1 expression vectors (shRNA1-NDRG1, shRNA2-NDRG1 and shRNA3-NDRG1) were transfected into OVCAR3 cells and the effects on biological processes were analyzed; transfection of an empty shRNA-NC vector served as a control. Depletion of NDRG1 at both the mRNA and protein levels was verified by both quantitative real-time RT-PCR and western blotting, respectively. NDRG1 expression was most significantly reduced by shRNA1-NDRG1 (Fig. 1B). Therefore, we chose to use shRNA1-NDRG1 for all subsequent experiments. We also generated a stable cell line (OVCAR3-shNDRG1) using this construct. Depletion of NDRG1 did not induce any observable morphological changes in the OVCAR3 cells (Fig. 1C).

OVCAR3-shNDRG1 cells displayed enhanced growth compared to OVCAR3-shNC cells at 72 h by MTT assay (P<0.05) (Fig. 1D). Consistent with this, OVCAR3-shNDRG1
cells formed more colonies than their OVCAR3-shNC counterparts (P<0.05) (Fig. 1E). These data indicate that NDRG1 plays a growth inhibitory role in OC.

**Cell migration and invasion are enhanced following NDRG1 depletion.** Previous studies have shown that NDRG1 likely plays a role in cancer metastasis (9,15). Here, we examined whether suppression of NDRG1 influences OVCAR3 cell migration and invasion. We performed wound healing assays to assess the effects on cell migration. Wound sizes in the OVCAR3-shNDRG1 cell group were significantly smaller than those in the OVCAR3-shNC group at both 24 and 48 h (P<0.05) (Fig. 2A). These data suggest that the NDRG1-depleted cells migrated faster than the control cells. These findings were complemented by data obtained from a Transwell assay. Significantly more OVCAR3-shNDRG1 cells migrated and invaded through the Transwell inserts compared to the OVCAR3-shNC control cells (P<0.05) (Fig. 2B and C). We also examined cell migration and invasion in another OC cell line, SKOV3. Similarly to what we found in OVCAR3 cells, NDRG1 knockdown in SKOV3 cells also resulted in enhanced migration and invasion (Fig. 2D). These data collectively indicate that suppression of NDRG1 promotes migration and invasion of OC cells in vitro.

**Suppression of NDRG1 promotes tumor progression in a xenograft model.** We next studied whether NDRG1 knockdown showed similar effects in vivo by using an OVCAR3 xenograft model in nude mice. Mice received intratumoral injections of OVCAR3-shNDRG1 (n=10) or OVCAR3-shNC (n=10) every two days. All mice in the OVCAR3-shNDRG1 group had significantly larger tumors than those in the OVCAR3-shNC group (P<0.05 from day 5 onwards; P<0.01 from day 13 onwards) (Fig. 3).
Overexpression of NDRG1 suppresses tumor cell proliferation and adhesion. Overexpression of NDRG1 in OVCAR3 cells significantly inhibited both cell proliferation (Fig. 4A and B) (P<0.05 at 48 h) and tumor cell adhesion (Fig. 4C) (P<0.05 at 48 h). We also induced NDRG1 overexpression in SKOV3 cells and found that this overexpression inhibited cell migration and invasion (Fig. 4D). These findings are consistent with our NDRG1 knockdown results. Taken together, the data showed that NDRG1 plays a growth suppressive and metastatic inhibitory role in OC.

Figure 2. Effects of NDRG1 knockdown on cell migration and invasion of OVCAR3 cells. (A) Cell migration was determined by wound healing assay. Cells (OVCAR3-shNC and OVCAR3-shNDRG1) were plated in 6-well plates to reach 100% confluency. A linear wound scratch was created with a 20-µl pipette tip. Distances between the edges of the wound were measured at 0, 24 and 48 h. (B) Representative images of in vitro cell migration and invasion following NDRG1 gene knockdown as determined by Transwell cell migration and invasion assays. Migrated and invaded cells were fixed and then stained with crystal violet. Images were captured using light microscopy (×100 magnification). (C) Migrated or invaded cells from five random fields per visual field (×200 magnification). (D) NDRG1 knockdown in SKOV3 cells also resulted in enhanced migration and invasion (*P<0.05 , **P<0.01). NDRG1, N-myc downstream regulated gene 1.

Figure 3. Suppression of NDRG1 expression by shRNA1-NDRG1 promotes tumor progression in a xenograft model. OVCAR3 cells were subcutaneously inoculated into one flank of each mouse. When the tumor size reached an average of 200 mm³ an intratumor injection of 5 µg shNDRG1 or 5 µg shNC in 30 µl PBS was administered every 2 days. The tumor volume was assessed before each injection using a digital caliper. Significant differences in the tumor volumes of both groups were observed beginning from day 5 of shRNA1-NDRG1 injection (P<0.05). NDRG1, N-myc downstream regulated gene 1; shRNA, short hairpin RNA.

Analysis of the cell cycle and apoptosis in NDRG1-overexpressing OVCAR3 cells. Cell cycle analysis showed that, compared to the control cells, NDRG1-overexpressing OVCAR3 cells displayed a significant increase in the percentage of cells in the G0/G1 phase (P<0.05), accompanied by a decrease in the percentage of cells in the S phase (P<0.05); there was no significant difference in the proportion of cells in the G2/M phase between the two groups (Fig. 5A).

Flow cytometry showed that apoptosis was increased in OVCAR3 cells transfected with pcDNA3.1(+)/NDRG1 when compared to control cells transfected with the empty vector (P<0.05) (Fig. 5B).

Overexpression of NDRG1 increases expression of p21 and p53. To assess downstream changes, we examined protein expression of NDRG1, p21 and p53 by western blotting following overexpression of NDRG1 in OVCAR3 cells. Both p21 and p53 were significantly increased in the pcDNA3.1(+)/NDRG1-transfected cells when compared to the control cells (P<0.01) (Fig. 6).

Discussion

NDRG1 has been shown to play a role both in normal biological processes, such as cell differentiation (20) and disease pathogenesis, including hereditary motor and sensory neuropathy Lom (HMSNL) (21) and carcinogenesis (22). Importantly, functional studies in tumor cells have yielded conflicting results concerning the role of NDRG1 in modulating tumor growth and metastasis. Most current reports indicate that it functions as a tumor suppressor in a number of cancers, including...
breast (9), prostate (10-12), pancreatic (7,8,13), cervical and endometrial cancer (14,23). However, in other types of tumors, for example, hepatocellular carcinoma, NDRG1 may actually have a growth-promoting role (17,18).

Knockdown of NDRG1 significantly enhanced cell proliferation, colony formation, migration, invasion and differentiation in most in vitro cell line studies. These include studies in pancreatic (8), cervical (24), prostate (11), lung (11), gastric (25) and colon cancer (15,26). Consistent with this,
finding, overexpression of NDRG1 has been shown to inhibit proliferation, migration, invasion and differentiatiation (7,26,27).

In the present study, we used a combination of shRNA-mediated knockdown and overexpression to examine the function of NDRG1 in OC both in vitro and in vivo. We found that depletion of NDRG1 in OVCAR3 cells promoted xenograft growth in nude mice. These findings are in line with overexpression studies that found decreased xenograft growth when NDRG1 levels were increased (7,26,28). Additionally, we found that knockdown of NDRG1 significantly enhanced cell proliferation, colony formation, migration and invasion of OVCAR3 cells. Furthermore, overexpression of NDRG1 in OVCAR3 cells significantly inhibited their proliferation, adhesion and progression through cell cycle arrest and apoptosis. Our findings in OVCAR3 cells were supported by similar data obtained in SKOV3 cells (as presented in this study) as well as in HO8910-PM cells (14). Taken together, these data suggest that NDRG1 functions as a tumor suppressor in OC. However, its exact role in this disease clearly requires further investigation.

We also showed that p21 and p53 were induced following NDRG1 overexpression. These data suggest that p21 transcription may be increased in a p53- and NDRG1-dependent manner in OVCAR3 cells. Recent reports have shown that mutant p53 can retain the ability to transactivate p21 (29,30), which is in contrast to previous reports showing that only wild-type p53 induces p21 expression (31,32). Importantly, p21 expression does not necessarily reflect the status of p53 in these cells.

In conclusion, we showed that NDRG1 suppresses OC cell growth and migration. It may also suppress the metastasis of OC, and further clinical investigation of its role in human tumors is warranted.

References