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ORIGINAL ARTICLE

N-myc downstream-regulated gene 2 promotes proliferation of HO-8910 ovarian cancer cells

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Abstract

Objective To investigate N-myc downstream-regulated gene 2 (NDRG2) expression in ovarian cancer cells and its potential usefulness as a diagnostic marker and/or target for therapeutic intervention.

Methods Human *NDRG2L/S* gene was obtained by revers-transcription polymerase chain reaction (RT-PCR). Sequence analysis confirmed the identity of *NDRG2L/S* gene, which was then inserted into a eukaryotic vector pLNCX2, which was in turn transfected into *NDRG2* gene-negative HO-8910 cells. Flow cytometry (FCM) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay were conducted to determine the proliferation rate of HO-8910 cells. Cisplatin resistance of HO-8910 cells transfected with pLNCX2-NDRG2L/S was evaluated by FCM. Tumors were generated in female nude mice by subcutaneous injection of HO-8910 cells.

Results *NDRG2* gene was isolated and its expression vector was successfully constructed. *NDRG2* expression positively correlated with the proliferation of HO-8910 cells. *NDRG2L/S* promoted tumorigenicity in HO-8910 cells.

Conclusion The present study identified a novel function of *NDRG2L/S* gene and demonstrated its involvement in the promotion of ovarian cancer cell proliferation and enhancement of cisplatin resistance in HO-8910 cells. Future studies are warranted to determine the relationship between *NDRG2* upregulation and ovarian cancer progression.

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Key words: N-myc downstream-regulated gene 2 (NDRG2); ovarian cancer; HO-8910 cell; MTT; cisplatin

Ovarian cancer is the fifth most common cancer in females and the leading cause of mortality related to gynecological malignancies [1–2]. It is the second most common gynecological cancer following cancer of corpus uteri, with 21,980 and 67,000 new cases reported in the United States and Europe in 2014 [3] and 2008 [4], respectively. The global incidence rate of ovarian cancer involves 225,500 new cases and 140,200 deaths every year, including 14,030 deaths in the United States alone [5]. As ovarian carcinoma presents nonspecific symptoms and is often asymptomatic until later stages, majority of patients are not diagnosed until they reach advanced stages of the disease [6–7]. Ovarian cancer is a lethal gynecological malignancy, with more than 70% of women presenting advanced stage disease [8]. Standard of care for ovarian cancer is surgical debulking, followed by combination treatment with platin-based drugs such as carboplatin and paclitaxel [9]. Cisplatin was first approved by the Food and Drug Administration (FDA) for the treatment of ovarian cancer in 1978 [10]. Some evidence exists to support the success of neoadjuvant chemotherapy in women who present with advanced, unresectable primary ovarian cancer, followed by interval debulking; however, some results also suggest that this approach imparts little or no benefit [11]. Most women initially respond to these chemotherapeutic drugs, but the majority would relapse within 2 years, ultimately developing broad chemoresistance [12]. Despite new treatments, no significant changes in long-term outcomes have been reported in the past 30 years, and more than 60% of advanced stage patients develop recurrent disease [13].

NDRG2, a member of the *N-myc* downstream-regulated gene family, belongs to the alpha/beta hydrolase superfamily. It was first cloned from a normal human brain cDNA library by subtractive hybridization (GenBank Accession No. AF159092) and is regarded as a tumor suppressor gene transcriptionally repressed by c-Myc [14–16]. The human *NDRG2* gene, located at chromosome 14q11.2, comprises 16 exons and 15 introns and encodes

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for a 41-kDa protein. Two isoforms of *NDRG2* have been previously described, one of which contains a 42-bp insertion in the mRNA owing to alternative splicing that results in a protein carrying additional 14 amino acid residues[17]. In this study, these isoforms were isolated and named as NDRG2L and NDRG2S. The biological significance of these isoforms is currently unknown. It has been proposed that *NDRG2* is a candidate tumor suppressor gene and its expression is generally low or undetected in various tumors and tumor cell lines. To date, no report has described the relationship between *NDRG2* expression and ovarian cancer.

The objective of this study was to investigate *NDRG2* function in the ovarian cancer cell line HO-8910 and explore the effects of *NDRG2* upregulation on cisplatin resistance in HO-8910 cells. In this study, the human *NDRG2L/S* gene was obtained by reverse-transcription polymerase chain reaction (RT-PCR) and subjected to sequence analysis. Furthermore, a retroviral vector NDRG2 expression system was used to verify the effects of pLNCX2-NDRG2L/S on the proliferation of HO-8910 cells and growth of tumor in a nude mouse model.

Materials and methods

Cell culture and gene transfection

HO-8910 cells were cultured in Dulbecco's modified Eagle's medium (HyClone, USA) supplemented with 10% fetal bovine serum (HyClone), 100 U/mL penicillin, and 1 × penicillin-streptomycin (100 U/mL and 100 μg/mL, respectively) (Invitrogen, USA). Plasmids were introduced into cells using a pLNCX2 retrovirus vector (BD, USA) system, as per the manufacturer's protocol. The transfected cells were selected by G418 and continuously cultured until harvest and subsequent analysis.

RT-PCR and real-time quantitative RT-PCR (qRT-PCR)

Regular RT-PCR and qRT-PCR were performed as previously described on an ABI PRISM 7300 detection system (ABI, USA) using the primers listed in Fig. 1. The RT-PCR reactions were repeated at least thrice.

Western blotting

For western blotting, cells were lysed using 20 mM Tris–HCl (pH 8.0), 5% glycerol, 138 mM sodium chloride (NaCl), 2.7 mM potassium chloride (KCl), 1% NP-40, 20 mM sodium fluoride (NaF), 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM sodium orthovanadate, 5 mg/mL leupeptin, 1 mg/mL pepstatin, and 1 mM dithiothreitol. The extracted proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) before transferring onto a polyvinylidene fluoride (PVDF) membrane, followed by incubation with anti-NDRG2 (ab57429, Abcam, USA) and anti-β-actin antibodies (Santa Cruz Biotechnology, USA).

Xenografting of ovarian cancer cells and tumor development in mice

Tumors were generated in nude mice (Vital River, China) by a subcutaneous injection of HO-8910 cells [5×10^5 cells in 100 μL of phosphate-buffered saline (PBS)] into the right dorsum of each mouse (10 mice in each group and a total of three groups). Tumor measurements were converted to tumor volume (V) using the formula ($L \times W^2 \times 0.52$), where L and W were the length and the width, respectively. Tumor growth was measured once every 2 days using a Vernier caliper. The mice were sacrificed on day 38, and the number and size of each tumor were macroscopically quantified. Macroscopic tumor images were acquired with a Canon camera and processed with Adobe Photoshop CS Version 8.0. All procedures were performed according to animal welfare and other related ethical regulations approved by the Institutional Animal Care Committee of Medical College at Xiamen University.

Data analysis and statistics

Data were presented as the mean ± standard deviation, as indicated for each figure. Statistical comparisons between groups were performed using the Student's *t*-test. A value of $P < 0.05$ was considered to indicate statistically significant differences.

Results

Modulation of NDRG2 expression by pLNCX2-NDRG2 in HO-8910 cells

To generate *NDRG2* mRNA, we cloned the *NDRG2* gene by RT-PCR (Fig. 1a). The pLNCX2-NDRG2 eukaryotic expression vector was obtained and confirmed by DNA sequencing (Fig. 1b). To determine the role of *NDRG2*, we used the HO-8910 cell line as the experimental model, as HO-8910 cells exhibit low endogenous *NDRG2* levels. The cells were infected with pLNCX2-NDRG2, and western blotting was used to evaluate NDRG2 upregulation. In comparison with the pLNCX2 group (negative control), cells treated with pLNCX2-NDRG2 showed upregulated expression of NDRG2 (Fig. 1c).

Proliferative effects of *NDRG2* overexpression on HO-8910 cells

To investigate the relationship between HO-8910 cells and *NDRG2* expression, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and colony formation assays were performed. The MTT

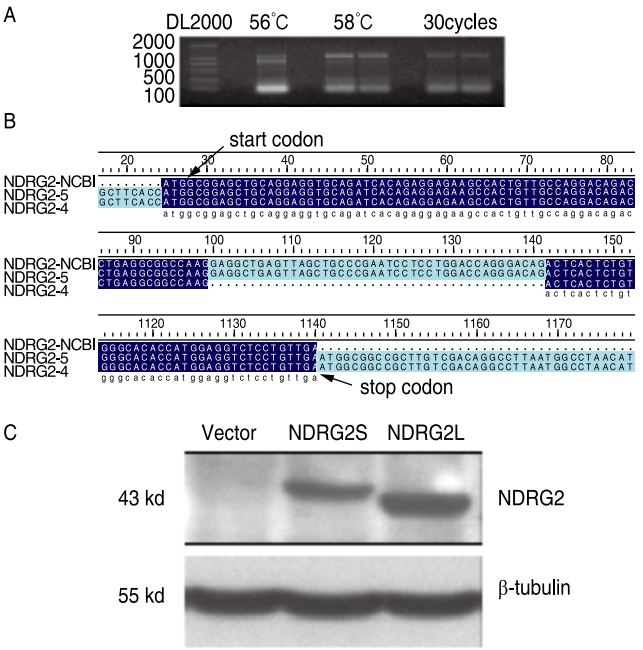

Fig. 1 Overexpression of NDRG2 in HO-8910 cells. (A) NDRG2 gene was cloned by RT-PCR; (B) The sequence of pLNCX2-NDRG2 eukaryotic expression vector was confirmed by DNA sequencing; (C) Western blot analysis of NDRG2 in HO-8910 cells infected with pLNCX2-NDRG2. Equal amounts of proteins were subjected to western blot analysis.

assay was designed with an OD gradient and infection time gradient (days 2, 4, and 6). We infected the cells with pLNCX2, pLNCX2-NDRG2L, or pLNCX2-NDRG2S; approximately 6 days later, the proliferation rate of the groups treated with pLNCX2-NDRG2L and pLNCX2-NDRG2S was significantly different from that of the control (Fig. 2a). Next, we assayed the contribution of pLNCX2-NDRG2L and pLNCX2-NDRG2S to colony formation in HO-8910 cells. Cells from each group were incubated for 2 weeks, followed by cell number enumeration. As shown in Fig. 2b, the colony formation ratio was significantly increased to 23.6% in cells treated with pLNCX2-NDRG2S as compared with that in the control group. In contrast, no obvious difference in the colony formation ratio was observed in the pLNCX2 group. These data revealed that NDRG2 overexpression promotes the proliferation of HO-8910 cells. Third, detection of the cell cycle changes by flow cycometry (FCM).

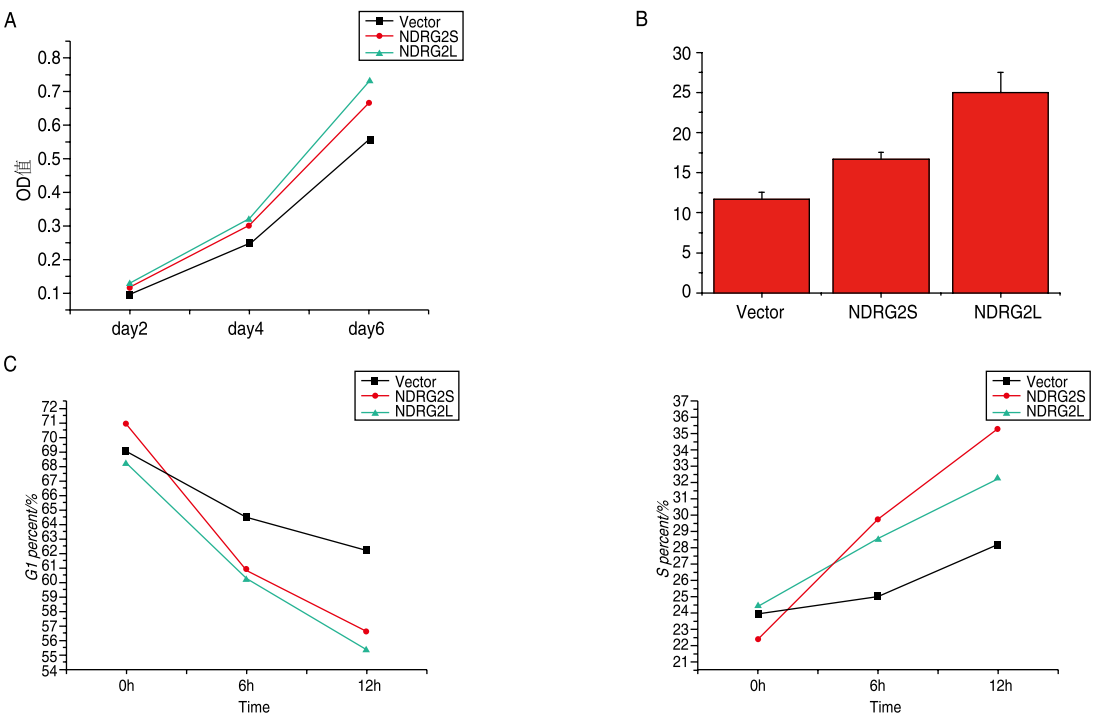

Fig. 2 NDRG2 overexpression promotes HO-8910 cell growth. (A) Time-dependent promotion of HO-8910 cell proliferation following pLNCX2-NDRG2-4 and pLNCX2-NDRG2-5 infection. After pLNCX2-NDRG2-4 and pLNCX2-NDRG2-5 infection, the cells were incubated for different time periods (2, 4, and 6 days). Cell proliferation was quantified using the MTT assay; (B) The effect of pLNCX2-NDRG2 on the colony formation ability of HO-8910 cells was examined. After pLNCX2-NDRG2-4 and pLNCX2-NDRG2-5 infection, the cells were incubated for approximately 10 days until colony formation was observed. Only the clearly visible colonies (diameter > 50 μm) were counted; (C) Detection of cell cycle changes by FCM.

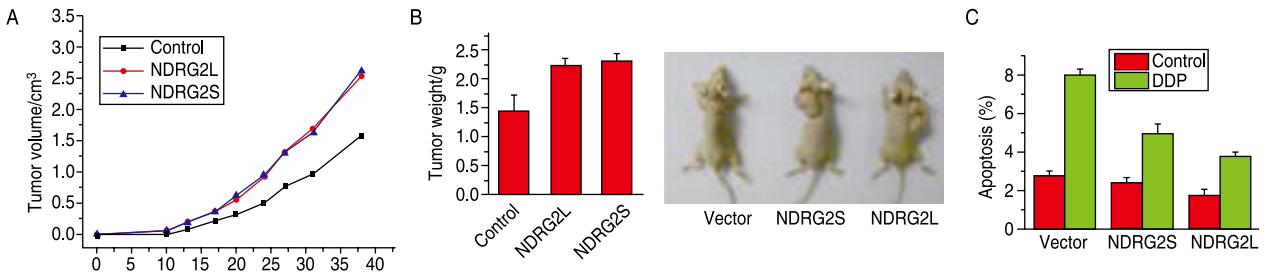

Fig. 3 Effects of pLNCX2-NDRG2 on the growth of HO-8910 cells xenografted into mice. (A) Tumor growth curve. Tumor growth was assessed every 3 days until day 38 by measuring two perpendicular diameters and calculating the volume in cubic centimeter. Statistical analysis was performed with values obtained on day 38 using one-way analysis of variance (ANOVA) and Student's *t*-test. ** indicates $P < 0.01$ as compared to the control; (B) Representative images of xenografted tumors; (C) *NDRG2* promotes resistance of HO-8910 cell lines to cisplatin (DDP)-induced apoptosis.

Promotion of tumor growth in a nude mouse model by intratumoral pLNCX2-NDRG2 injection

To investigate the effects of NDRG2 expression on the tumor growth *in vivo*, tumors were generated in nude mice by subcutaneous injection of HO-8910 cells (5×10^5 cells in 100 μL of PBS) that had been infected with pLNCX2, pLNCX2-NDRG2L, or pLNCX2-NDRG2S into the right dorsum of each mouse. As shown in Figure 3a and 3b, the tumors from pLNCX2-NDRG2L and pLNCX2-NDRG2S groups showed sustained and significant growth (mean tumor volume on day 38 of 2.80 and 1.45 cm^3, respectively). NDRG2 promoted the resistance of HO-8910 cells to cisplatin (DDP)-induced apoptosis (Fig. 3c).

Discussion

The expression of *NDRG2* is ubiquitous and particularly high in normal human tissues, while the bone marrow, testis, peripheral blood, and placenta exhibit relatively low *NDRG2* expression. *NDRG2* expression is almost undetectable in human pancreatic cancer, hepatocellular carcinoma [18], thyroid cancer, colorectal cancer [19], gastric cancer [20], and some human cancer cell lines such as those of breast, stomach, and colon [21]. The distinct expression patterns between normal and neoplastic tissues and cell lines suggest that *NDRG2* is a differentiation-related gene and may play a vital role in homeostasis. *NDRG2* has been identified as a prognostic marker in gastric cancer because of its significantly decreased expression, which, in turn, has been strongly associated with poor prognosis and low survival rates [22]. However, no report has described the relationship between NDRG2 gene and ovarian cancer.

Previous studies have shown that *NDRG2* overexpression reduces glioblastoma proliferation *in vitro* [23], while *NDRG2* silencing was found to enhance the proliferation of colon cancer and gastric cancer cells *in vitro* [24]. Furthermore, *NDRG2* overexpression suppresses human liver cancer invasion and migration *in vitro* and reduces metastasis *in vivo* [25]. These findings suggest that *NDRG2* may be an important malignancy factor. In our research, the MTT assay and FCM results showed that the upregulation in *NDRG2* expression increased the proliferation rate of HO-8910 cells. In addition, *NDRG2* facilitated the transition of HO-8910 cells from G0/G1 phase to S phase. *NDRG2* promoted the resistance of HO-8910 cells to cisplatin (DDP)-induced apoptosis. This finding is contradictory to the results of a previous study on the role of *NDRG2* in tumor metastases. The mechanism underlying this observation remains unknown.

In summary, the present study demonstrates for the first time that *NDRG2* overexpression induces proliferation in ovarian cancer HO-8910 cells *in vitro* and *in vivo*. Therefore, *NDRG2* gene may be a promising target for the development of novel therapeutics and may potentially play an important role in the prevention and treatment of ovarian cancer.

Conflict of interest

The authors declare no conflict of interest.

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