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Expression of NDRG2 in esophageal squamous cell carcinoma

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N-Myc downstream-regulated gene 2 (NDRG2), a new member of the N-Myc downstream-regulated gene family, has been found to be a differentially expressed gene involved in a variety of cancers. The present study aimed to investigate the expression of NDRG2 in esophageal squamous cell carcinoma (ESCC). Immunohistochemistry was performed in 154 samples from patients with ESCC to detect the expression level of NDRG2 and C-MYC. Results indicated that the expression level of NDRG2 in the cancer samples was significantly lower than that in normal tissues; the trend of C-MYC was the reverse. The Wilcoxon–Mann–Whitney test showed significant difference in the expression of NDRG2 in patients with different T stage, TNM stage, and differentiation degree of cancers (P = 0.036, 0.031, 0.001, respectively). Patients in stages I and II were followed up for 5 consecutive years and Kaplan–Meier survival analysis demonstrated that the survival time of ESCC patients with high expression of NDRG2 was longer than those with low expression during the 5-year follow-up period (P = 0.0018). Cox regression analysis indicated that low expression of NDRG2, cancer stage of pT1, and distant organ metastasis (pM1) were the independent poor prognostic factors of ESCC (P = 0.004, 0.019, 0.0013, respectively). Furthermore, up-regulation of NDRG2 was introduced to ESCC cell lines (EC9706 and EC109) by plasmid transfection. *In vivo* and *in vitro* studies indicated that overexpression of NDRG2 markedly reduced proliferation and promoted the apoptosis of EC9706 and EC109 cells. In summary, our results demonstrated that NDRG2 played an important role in the proliferation of ESCC cells and the expression of NDRG2 in ESCC was closely related with the prognosis. (*Cancer Sci* 2010; 101: 1292–1299)

Esophageal squamous cell carcinoma (ESCC) is one of gastrointestinal cancers with extreme malignance. Epidemiological evidence indicates ESCC is the sixth most common cause of cancer-related deaths in the world and it is the seventh most common cause of death in the USA.[1,2] China is also one of high-risk areas of ESCC, and higher morbidity and mortality of ESCC is presented in China than in Europe and North America.[3,4] Although great progress has been made in the surgical treatment, chemotherapy, and radiotherapy of ESCC, and the survival time has been improved in ESCC patients, the 5-year survival rate of ESCC patients is still as low as 20–30%.[1] Nowadays, gene therapy is a novel therapeutic mode and trend in the treatment of ESCC patients. It has been confirmed that several genetic abnormalities and gene mutations are involved in the pathogenesis of ESCC.[5–7] So, more studies on gene therapy in ESCC are needed to improve the prognosis of ESCC.

Our group initially cloned human N-Myc downstream-regulated gene 2 (NDRG2) from a normal human brain cDNA library and found that NDRG2 was located on chromosome 14q11.2. The *NDRG2* gene contains 16 exons and 15 introns and its full mRNA is 2024 bp in length. It encodes a protein consisting of 357 amino acids with a predicted molecular weight of about 40 kDa.[8,9] The *NDRG2* gene is one of the *NDRG* gene family members and currently four members in the human NDRG family have been found: NDRG1, NDRG2, NDRG3, and NDRG4. Highly conserved sequence of NDRG family members was observed from lower organisms to higher organisms, suggesting that they may play important roles in the cellular life processes.[10,11]

Our group had found high expression of NDRG2 in the central nervous system such as brain gray matter, white matter, and cranial nerve nuclei. In addition, salivary gland epithelial cells and skeletal muscle cells, belonging to terminally differentiated cells such as neurons, also have relatively high expression of NDRG2. However, bone marrow stem cells and spermatogonia were found to have low expression of NDRG2, which was also noted in the embryonic brain, heart, liver, kidney, and lung compared to adult organs.[10,12–14] Recently, studies have observed that the expression of NDRG2 in varieties of cancers such as colon, gastric, and liver was different from that in normal tissues.[15–18] These findings suggest that the expression of NDRG2 is negatively related with cell proliferation, especially with the proliferation cancer cells.

In the present study, the expression level of NDRG2 and C-MYC was firstly detected in 154 samples from patients with ESCC and the relationship between expression of NDRG2 and survival time during the 5-year follow-up period was evaluated. In addition, overexpression of NDRG2 was introduced to EC9706 and EC109 cells by plasmid transfection, and *in vitro* as well as *in vivo* studies were conducted to investigate the role of NDRG2 in the proliferation and apoptosis of EC9706 and EC109 cells.

Materials and Methods

Patients and collection of samples. A total of 154 patients with primary ESCC, who underwent surgery at the Department of Thoracic Surgery at Tangdu Hospital, were recruited into the present study from 2000 to 2003. The mean age was 58 years (range, 40–72 years) with 50 women and 104 men. Cancer tissues and normal tissues, which were at least 3–4 cm away from the cancer, were obtained from the patients. Semiquantitative reverse transcription-PCR and western blot analysis were performed in fresh samples from 10 patients. In addition, cancer

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and normal tissues from 154 patients were embedded in paraffin and cut into sections for immunohistochemical analysis. The median follow-up period was 23.7 months (range, 3–65 months). Patients' characteristics such as gender, age, location of tumor, stage of disease, and histopathological factors were obtained from the medical records. Patients' features are summarized in Table 1. All cancers were clinicopathologically confirmed to be ESCC. All the patients were staged based on the TNM staging system. Of the 154 patients, 24 (15.6%) had T1 stage ESCC, 47 (30.5%) T2, 49 (31.8%) T3, and 34 (22.1%) T4. Tissues were fixed in 10% formaldehyde, embedded in paraffin, cut into 4-μm sections, and mounted on slides. All patients gave informed consent to use excess pathological specimens for research purposes. The protocols used in the study were approved by the hospital's Protection of Human Subjects Committee. The use of human tissues in this study was approved by the institutional review board of The Fourth Military Medical University and was done in accordance with international guidelines.

Immunohistochemical staining. Immunohistochemistry was performed as described previously.[15] Avidin–biotin–peroxidase method was used for immunohistochemical assay. All sections were deparaffinized with xylene and dehydrated with gradient alcohol followed by inactivation of endogenous peroxidase activity by 0.5% H_2O_2 in methanol for 10 min. Nonspecific binding was blocked by incubation with 10% normal goat serum in phosphate-buffered saline (PBS) for 1 h at room temperature. Then, slides were incubated with mouse anti-NDRG2 antibody (1:100; Abnova, Walnut, CA, USA) or anti-C-MYC (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight, followed by biotinylated goat antimouse IgG (1:400; Sigma, St. Louis, MO, USA) or goat antirabbit IgG (1:400; Sigma) for 1 h at room temperature. Then streptavidin–biotin–peroxidase-complex assay was performed. A brown color was indicative of NDRG2 and C-MYC expression. Peroxidase activity was developed by incubating with 0.1% 3,3-diaminobenzidine (Sigma) in PBS with 0.05% H_2O_2 for 5 min at room temperature.

Table 1. Correlation between clinicopathological characteristics and N-Myc downstream-regulated gene 2 (NDRG2) expression

	NDRG2 (−/+)	NDRG2 (++)	NDRG2 (+++)	*P*-values
Age (years)				
<60	48	17	11	0.235
≥60	38	34	6	
Gender				
Male	56	36	12	0.487
Female	30	15	5	
Location				
Upper	20	14	2	0.737
Middle	38	18	8	
Lower	28	19	7	
pT				
pT_1	18	3	3	0.036
pT_2	19	20	8	
pT_3	27	18	4	
pT_4	22	10	2	
pN				
pN_0	41	24	9	0.837
pN_1	45	27	8	
pM				
pM_0	76	45	15	0.980
pM_1	10	6	2	
TNM				
I + II	36	29	11	0.031
III + IV	50	22	6	
Histologic				
1	13	11	11	0.001
2	43	29	5	
3	30	11	1	

Immunohistochemical analysis. Sections without the primary antibody were used as negative controls. Colorectal cancer samples that were shown previously[15] to have immunoreactivity with NDRG2 antibody were used as positive controls to confirm the NDRG2 expression. NDRG2-positive samples were defined as those showing a cytoplasmic pattern of lesional tissue. The slides were evaluated independently by two pathologists who were blind to the study. Any disagreement was resolved by consensus after joint review. Expression of NDRG2 was evaluated as the percentage of positive cells, and by staining intensity as described previously. The percentage of positive cells was evaluated quantitatively and scored as 0 for staining of ≤1% of total cells counted, 1 for staining of 2–25%, 2 for staining of 26–50%, 3 for staining of 51–75%, and 4 for staining of >75% of the cells examined. Intensity was graded as follows: 0, no signal; 1, weak; 2, moderate; and 3, strong staining. A total ''staining score'' of 0–12 was calculated and graded as negative (−, score 0–1), weak (+, score 2–4), moderate (++, score 5–8), or strong (+++, score 9–12).[19,20]

Statistical analysis. The relationship between NDRG2 expression levels and clinicopathological factors was analyzed using the Wilcoxon–Mann–Whitney test.[21,22] The overall survival time of stage I and II patients was defined as the time from surgery to death due to cancer, for patients who had not been treated by radiotherapy and chemotherapy. The Kaplan–Meier method was used to determine the cumulative probability of survival, and data were analyzed with log-rank test. Multivariate statistical analysis was done using the Cox regression model to investigate the effects of patients' characteristics (NDRG2 status, gender, age, location, extent of primary tumor, nodal status, metastasis,and histological grade) on overall survival. A score was assigned to each variable for the Cox regression analysis. Changes of cell cycle of EC9706 and EC109 cells untreated, transfected with pcDNA3.1, and pcDNA3.1-NDRG2 were analyzed by ANOVA. The apoptotic rates of EC9706 and EC109 cells untreated, transfected with pcDNA3.1, and pcDNA3.1-NDRG2 were analyzed by using the Wilcoxon–Mann–Whitney test. A value of $P < 0.05$ was considered statistically significant.

RNA extraction and semiquantitative RT-PCR. Total RNA was extracted from the cancer tissues by the acid guanidinium thiocyanate-phenol-chloroform extraction method. Total RNA was dissolved in 0.1% diethylpyrocarbonate-treated water. The amount of RNA was determined by spectrometrically measuring absorbance at 260 nm. The NDRG2 and C-MYC forward and reverse primers were 5′-GCCCAGCGATCCTTACCTACC-3′ and 5′-GGCTGCCCAATCCATCCAACC-3′, and 5′-GGAGGAACAAGAAGATGAGGAAG-3′ and 5′- AGGACCAGTGGGCTGTGAGG-3′ respectively. The housekeeping gene β-actin was used as internal standard for quantifying RNA levels. The β-actin forward and reverse primers were 5′-ATCATGTTTGAGACCTTCAACA-3′ and 5′-CATCTCTTGCTCGAAGTCCA-3′, respectively. PCR was performed in a GeneAmp PCR system 2400 thermocycler (Perkin Elmer, Norwalk, CT, USA). The cycling conditions for PCR were 30 cycles of denaturation (94°C for 40 s), annealing (55°C for 30 s), and extension (72°C for 40 s). PCR products were loaded onto 1.5% agarose gel and electrophoretically separated. The gel was then visualized under ultraviolet light following ethidium bromide staining.[9,15] Each experiment was carried out in triplicate.

Western blot analysis. Total tissue proteins were extracted in lysis buffer and then centrifuged at 12 000*g* for 5 min at 4°C. The supernatants were collected, and protein concentrations were determined, using Bio-Rad protein assay dye reagent

(Bio-Rad, Hercules, CA, USA). Aliquots (50 mg) of whole protein lysates were loaded onto sodium dodecyl sulfate–polyacrylamide (10%) gels for electrophoresis. For western blotting analysis, proteins were transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). The membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 and incubated with primary antibody (NDRG2 and C-MYC) overnight at 4°C. Finally, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Promega, Madison, WI, USA) for at least 1 h at room temperature and detected using the ECL method (Amersham Biosciences).[9,23]

Cell culture. Human ESCC cell lines EC109, EC9706, EC8712, KYSE150, KYSE70, and KYSE510 were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained as recommended. All ESCC cell lines were grown in 1640 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (Atlanta Biologicals), 10 U/mL penicillin, and 10 U/mL streptomycin, at 37°C in a humidified atmosphere containing 5% CO_2.[21,24]

Plasmid construction and cell transfection. The human *NDRG2* gene was inserted into the Bam H I and EcoR I sites of the pcDNA3.1 (+) plasmid.[9,25] DNA sequencing was performed to confirm the integrity of NDRG2 sequence. When the cell fusion reached 80%, pcDNA3.1-NDRG2 and pcDNA3.1 (+) plasmids were transfected into esophageal cancer cell lines (EC109 and EC9706) according to the manufacturer's instructions (Lipofectamine 2000; Invitrogen, Carlsbad, CA, USA). About 48 h after transfection, the cells were incubated with G418 (600 μg/μL). Three weeks later, successfully transfected clones were selected and cell lines stably expressing NDRG2 were established. Western blotting assay was performed to detect transfection efficiency. The stable cell lines were maintained in 1640 medium containing 10% fetal bovine serum, 1% antibiotics, and 100 μg/μL G418.

Monolayer growth rate. Monolayer culture growth rate was determined as previously described[19,26] by conversion of MTT (Sigma Chemical Co., St. Louis, MO, USA) to water-insoluble formazan by viable cells. Approximately 3000 cells in 200 μL medium were plated in 96-well plates and grown under normal conditions. Cultures were assayed every day for 7 days and absorbance values were determined with an enzyme-linked immunosorbent assay reader (DASIT, Milan, Italy) at 490 nm. Each experiment was preformed in triplicate.

Plate clone formation assay. Plate clone formation assay was as described previously.[19] For colony formation assays, 1×10^3 cells were seeded into 60-mm dishes with 5 mL DMEM supplemented with 10% fetal bovine serum (Sigma Chemical Co.) and 400 μg/mL G418 (Merck, Darmsdadt, Germany). After 14 days, the resulting colonies were rinsed with PBS, fixed with methanol at −20°C for 5 min, and stained with Giemsa (Sigma-Aldrich) for 20 min. Only clearly visible colonies (diameter >50 μm) were counted.

Soft agar colony formation assay. Soft agar colony formation assay was performed as previously described[19] to assess anchorage-independent growth, as a characteristic of *in vitro* tumorigenicity. Briefly, cells were detached and plated in 0.3% agarose with a 0.5% agarose underlay (1×10^4/well in six-well plates). The number of foci >100 μm was counted after 17 days. Each experiment was done in triplicate.

Tumorigenicity in nude mice. Tumorigenicity in nude mice was measured as described previously.[19] For tumorigenicity assay, four groups of five mice each were injected subcutaneously at a single site with stably transfected cells. Tumor onset was scored visually and by palpitation at the sight of injection by two trained members of the laboratory staff at different times on the same day. Average tumor size was estimated by physical measurement in cm of the excised tumor at the time of death. With the exception of mice with large tumor burdens, animals were killed 4 weeks after injection. Tumors were verified by H&E staining. Blocks were stored for further analysis.

Cell cycle analysis. Flow cytometric analysis was carried out as described previously.[19,26] Cells were seeded into 60-mm-diameter plates and grown in complete medium overnight. Then, the cells were maintained in serum-free medium for 48 h to synchronize the cells, and grown in complete medium for 24 h before harvesting. After washing with ice-cold PBS, cells were suspended in 0.5 mL of 70% alcohol and kept at 4°C for 30 min. The suspension was filtered through 50-mL nylon mesh, and the DNA content of stained nuclei was analyzed using a flow cytometer (EPICS XL; Coulter, Miami, FL, USA). The cell cycle was analyzed using Multicycle-DNA Cell Cycle Analyzed Software. The proliferation index (PI) was calculated as PI = (S + G2)/(S + G2 + G1). Each experiment was performed in triplicate.

Apoptosis detection with flow cytometer. Untreated and transfected EC9706, EC109 cells were trypsinized at 24, 48, and 72 h, washed with cold PBS and resuspended in PBS. Annexin V-FITC (BD Biosciences, San Jose, CA, USA) at a final concentration of 1 μg/mL and 250 ng of propidium iodide were added to the mixture containing 100 μL of cell suspension and binding buffer (BD Biosciences). The cells were vortexed and incubated for 15 min at room temperature in the dark, followed by incubation with 400 μL of binding buffer for flow cytometric analysis.

Results

NDRG2 and C-MYC expression in ESCC. NDRG2 protein expression in ESCC determined by immunohistochemistry was significantly decreased compared with that in normal epithelia (Figs 1A,2A,B), but there was no significant difference in the expression level of NDRG2 protein between normal and dysplasia esophagus tissues. NDRG2 was mainly localized in the cytoplasm of epithelial cells (Fig. 1a). C-MYC expression followed the opposite pattern, being abundant in poorly differentiated tumors compared with the well-differentiated tumors and normal esophagus tissue. C-MYC was localized in the nucleus of esophagus tumors (Figs 1(Be–h),2A,B). As shown in Figure 1(Aa–d) and Table 1, 154 ESCC patients were subdivided into the following three subgroups based on the expression levels of NDRG2, 86 with none to weak expression (55.8%; −/+), 51 with moderate to locally strong expression (33.1%; ++), and 17 with strong expression (11.1%; +++).

The correlation between expression level of NDRG2 and patients' characteristics, such as gender, age, location of cancer, stage of disease, and histopathological factors was investigated. NDRG2 protein expression was correlated with primary tumor (pT), tumor stage (TNM), and histological grade (Table 1; $P = 0.036$, 0.031, 0.001, respectively). And no correlation was observed between NDRG2 expression and age, gender, location, and pN and pM. Overall survival analysis using the Kaplan–Meyer method revealed that the prognosis of stage I and II patients with high or moderate NDRG2 expression was significantly better than those with none or weak NDRG2 expression (Fig. 1C; $P = 0.0018$).

The multivariate analysis showed that primary tumor (>pT_1), NDRG2 low-expression (−/+), and distant tumor metastasis (pM1) were independent poor prognostic factors of ESCC; however, age (>60 years), gender (male), location (midthoracic, lower), regional lymph node metastasis (pN1), and histological grade (>G2) were not related with the prognosis of ESCC (Table 2).

To further confirm these findings, RT-PCR and western blot analysis were performed in 10 freshly obtained ESCC tissues and adjacent normal tissues. We found that cancer tissues tended to show lower NDRG2 expression compared with the adjacent

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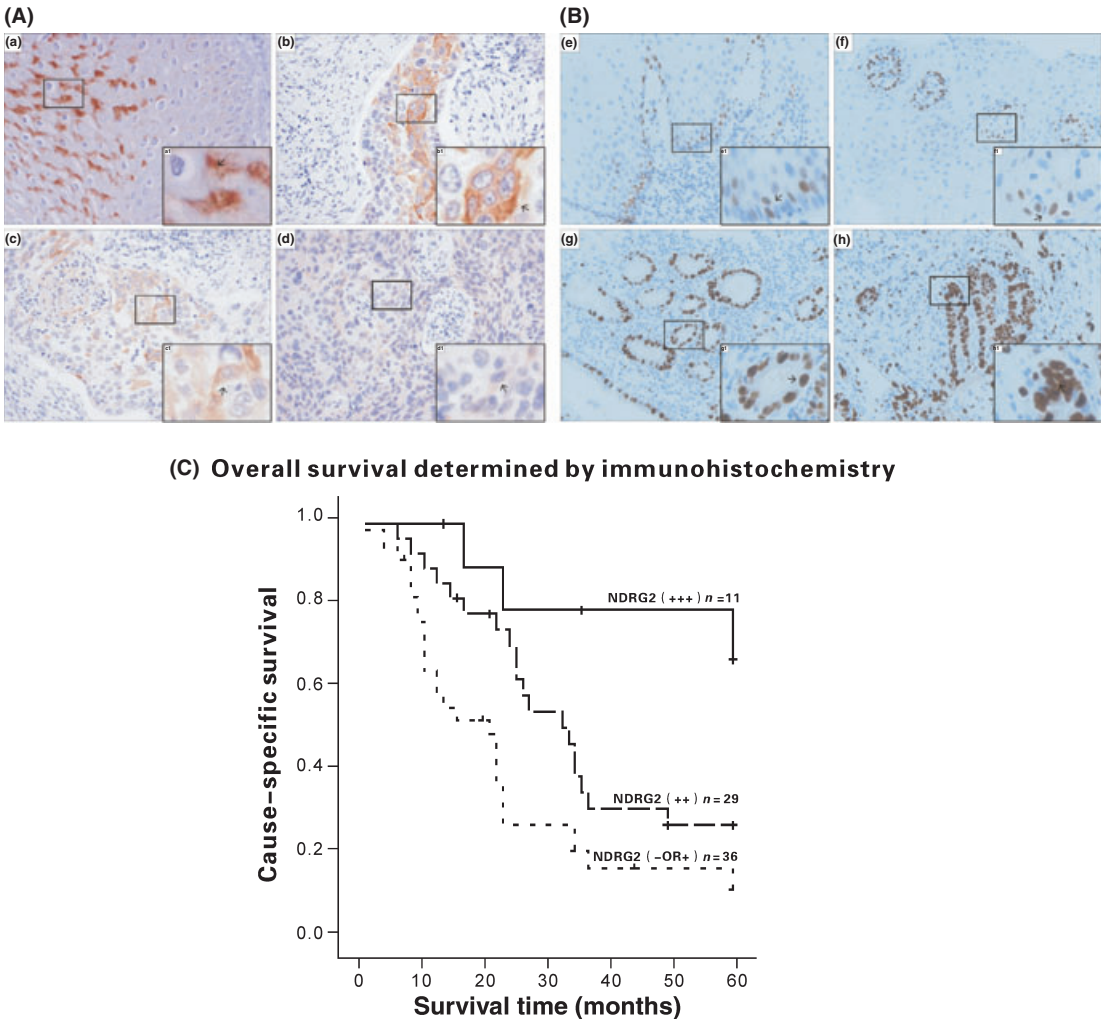

Fig. 1. (A) Immunohistochemical staining of N-Myc downstream-regulated gene 2 (NDRG2) in esophageal squamous cell carcinoma (ESCC) tissues. (a) Strong staining (+++) was observed in the normal esophageal epithelial cells, mainly in the epithelial cells, and no evident expression of NDRG2 was noted in cells of the germinal layer (×200). (b) Intermediate staining (++) of NDRG2 in ESCC tissues with high differentiation, mainly in the epithelial tissues (×200). (c) Weak staining (+) of NDRG2 in ESCC tissues with intermediate differentiation, mainly in the epithelial tissues (×200). (d) Negative staining (–) of NDRG2 in ESCC tissues with low differentiation (×200). (B) Immunohistochemical staining of C-MYC in esophageal carcinoma tissues. (e) Weak or Negative staining (–/+) was observed in the normal esophageal epithelial cells, mainly in the epithelial cells, and no evident expression of C-MYC was noted in cells of the germinal layer (×200). (f) Weak staining (+) of C-MYC in ESCC tissues with high differentiation, mainly in the epithelial tissues (×200). (g) Intermediate staining (++) of C-MYC in ESCC tissues with intermediate differentiation, mainly in the epithelial tissues (×200). (h) Strong staining (+++) of C-MYC in ESCC tissues with low differentiation (×200). (C) Cause-specific survival of patients determined by the immunoactivity of NDRG2. Cause-specific survival analysis using the Kaplan–Meyer method revealed that the stage I and II patients with relatively high expression of NDRG2 had a more favorable prognosis compared to those with low expression (P = 0.0018).

normal tissues in six samples, and the trend of C-MYC was the reverse (Fig. 2A,B), which was consistent with the findings in immunohistochemical analysis. However, no significant changes were found in the remaining four samples. The mRNA and protein expression of NDRG2 was determined in the human ESCC cell lines (EC109, EC9706, EC8712, KYSE150, KYSE70, and KYSE510). As shown in Figure 2(C), NDRG2 expression was observed in all ESCC cell lines and relatively low expression was noted in EC9706 and EC109 cell lines.

Effects of NDRG2 on cancer cell proliferation and growth as well as tumorigenicity. To up-regulate the expression of NDRG2 in human ESCC cells, the NDRG2-specific vector pcDNA3.1-NDRG2 was constructed.[16] EC9706 and EC109 were used for transfection because low expression of NDRG2 was observed in the two cell lines (Fig. 2C). After cell transfection and antibiotic screening for 6 weeks, the expression of NDRG2 in stably transfected cells was determined by western blotting assay. pcDNA3.1-NDRG2 was found to have increased expression of NDRG2 in EC9706 and EC109 cells, whereas no profound increase in expression of NDRG2 was observed in the pcDNA3.1-NC vector (negative control) (Fig. 3A). EC9702 and EC109 cells transfected with pcDNA3.1-NC or pcDNA3.1-NDRG2 were prepared for further cellular assays. When the growth curves of these cell lines were compared in medium containing 10% FCS, the curves for cells transfected with pcDNA3.1-NDRG2 were significantly lower than control cells ($P < 0.05$ on days 4–7; Fig. 3B) and cells transfected with pcDNA3.1-NC ($P < 0.05$ on days 4–7; Fig. 3B). To determine the effects of NDRG2 on the colony forming ability of ESCC cells, *in vitro* plate colony formation assays were performed. Compared to control cells and cells transfected with pcDNA3.1-NC, fewer colonies were observed in cells transfected with

Table 2. Cox multivariate analysis

Variables	Risk ratio (95% confidence interval)	*P*-values
Age (>60 years)	1.183 (0.780–1.794)	0.428
Gender (male)	0.839 (0.538–1.311)	0.441
Location (midthoracic)	0.659 (0.399–1.086)	0.102
Location (lower)	0.770 (0.458–1.296)	0.326
Primary tumor (>pT_1)	2.939 (1.193–7.241)	0.019
Regional lymph node metastasis (pN_1)	0.975 (0.591–1.608)	0.921
Distant metastasis (pM_1)	5.848 (1.990–17.189)	0.0013
Histologic grade (>G2)	0.752 (0.410–1.380)	0.358
NDRG2 (++)	0.616 (0.383–0.989)	0.045
NDRG2 (+++)	0.288 (0.123–0.675)	0.004

Values are mean ± SD, $n = 3$. NDRG2, N-Myc downstream-regulated gene 2.

pcDNA3.1-NDRG2 ($P < 0.01$, Fig. 3C). Additionally, the effect of NDRG2 on anchorage-independent colony formation in soft agar was evaluated as an additional *in vitro* assessment of tumorigenicity. Both colony number and size in soft agar were significantly reduced by NDRG2 up-regulation ($P < 0.01$, Fig. 3D). Collectively, our results indicated that overexpression of NDRG2 impaired the colony forming ability of EC9706 and EC109 cells *in vitro*. Then, *in vivo* tumor formation assays was performed in nude mice. We did not see any difference in the rate of tumorigenicity itself in NDRG2-transfected cells (5/5) compared to control cells (5/5). But the cancer size was significantly reduced in pcDNA3.1-NDRG2 groups compared to control and pcDNA3.1-NC groups (Fig. 3E, $P < 0.05$). Therefore, both *in vitro* and *in vivo* assays suggested that overexpression of NDRG2 might inhibit proliferation, growth, and tumorigenicity of ESCC cells.

Changes to cell cycle and apoptosis in esophageal squamous cell lines transfected with NDRG2. To further investigate the potential mechanism implicated in the suppressed growth of esophageal squamous cells by NDRG2 overexpression, FACS analysis was performed to study the effects of NDRG2 expression on cell cycle. The results showed that at 24 h after synchronization, 10.8% of EC9706 cells transfected with pcDNA3.1-NDRG2 were in the S-phase, while 37.3% of control cells and 34.6% of cells transfected with pcDNA3.1-NC ($P = 0.0002$) were in the S-phase. 12.8% of EC109 cells transfected with pcDNA3.1-NDRG2 were in the S-phase, while 32.2% of control cells and 29.6% of cells transfected with pcDNA3.1-NC ($P = 0.0018$) were in the S-phase.

Additionally, apoptotic rates of EC9706 and EC109 cells untreated, transfected with pcDNA3.1-NC, or pcDNA3.1-NDRG2 were analyzed by flow cytometer. The results revealed that there was no significant difference in apoptotic rate between

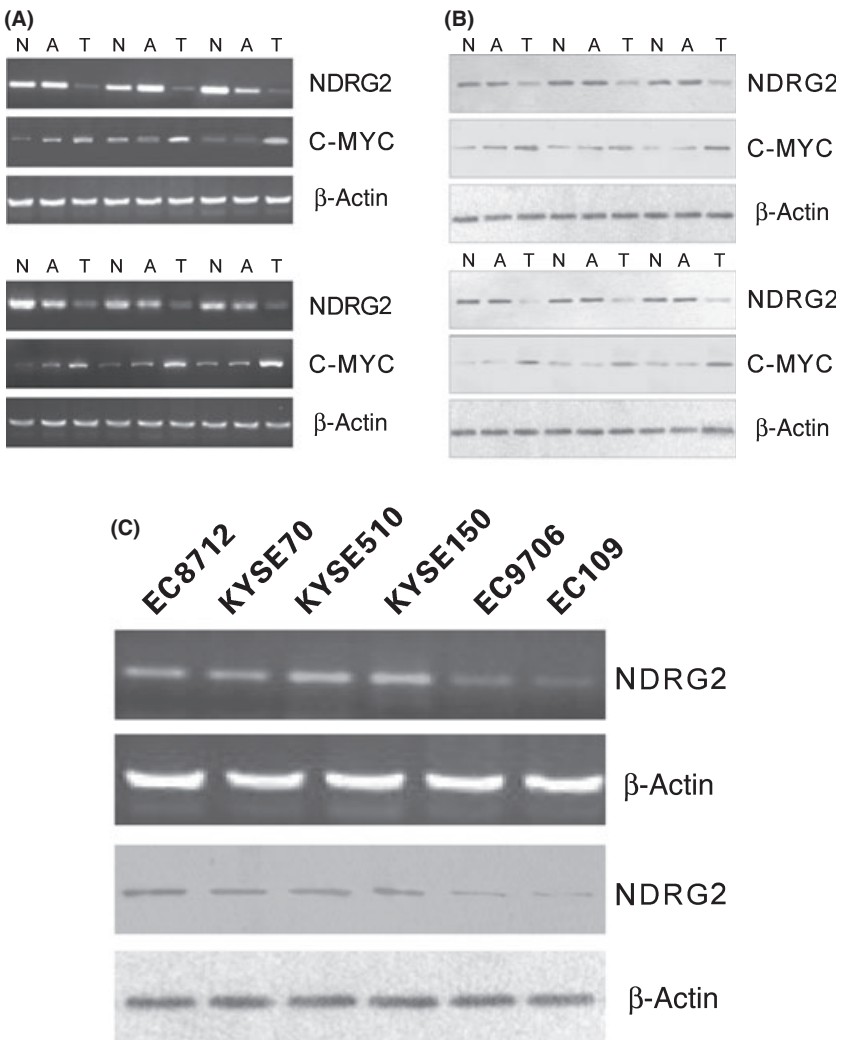

Fig. 2. Detection of N-Myc downstream-regulated gene 2 (NDRG2) and C-MYC expression in freshly obtained esophageal cancer tissues and cell lines by RT-PCR and western blot analysis. (A) mRNA expression of NDRG2 and C-MYC were detected with RT-PCR in esophageal squamous cell carcinoma (ESCC) tissues and its adjacent tissue as well as normal tissues. Profound changes were observed in six patients. A represents adjacent normal tissues; N represents distant normal tissues; T represents esophageal tumor tissues. β-Actin was used as an internal control. (B) Protein expression of NDRG2 and C-MYC were detected by western blotting in ESCC tissues and its adjacent tissue as well as normal tissues. Profound changes were observed in six patients. T represents esophageal tumor tissues, A represents adjacent normal tissues; N represents distant normal tissues; T represents esophageal tumor tissues. β-Actin was used as an internal control. (C). RT-PCR and western blotting assay were performed to detect the expression of NDRG2 mRNA and protein in esophageal cancer cell lines (EC109, EC9706, EC8712, KYSE150, KYSE70, KYSE510). β-Actin was used as an internal control.

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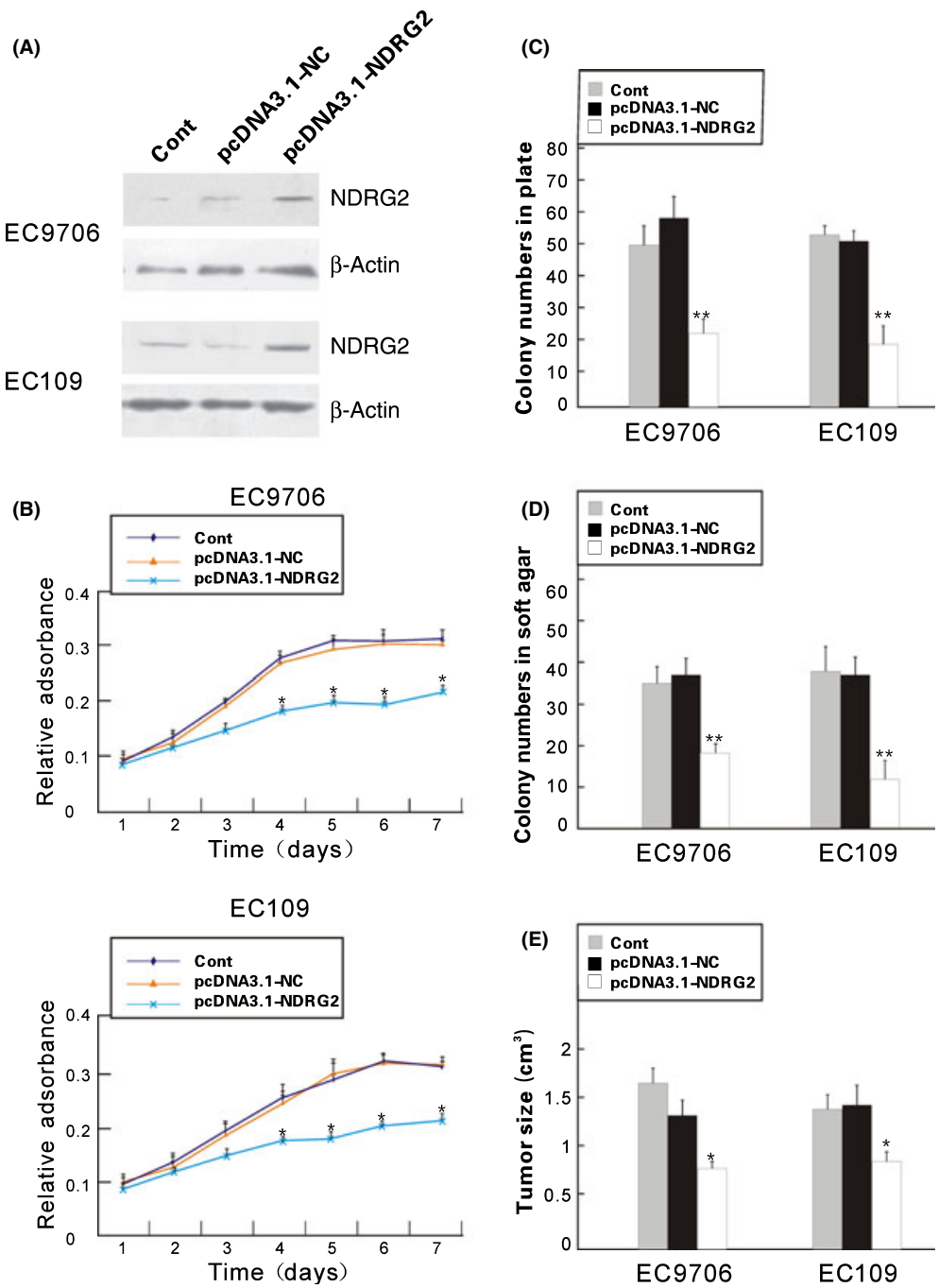

Fig. 3. Effects of N-Myc downstream-regulated gene 2 (NDRG2) on esophageal squamous cell carcinoma (ESCC) cell proliferation and tumorigenicity in nude mice. (A) The expression of NDRG2 in EC-7906 and EC 109 cells with NDRG2 transfection was evaluated by western blotting. β-Actin was used as an internal control. (B) Monolayer growth rates of EC-7906 and EC 109 cells transfected with NDRG2 were determined by MTT assay. Data are presented as the mean ± SEM from at least three separate experiments. $*P < 0.05$. (C) Plate clone formation assay. Data are presented as the mean ± SEM from at least three separate experiments and each experiment was preformed in triplicate. $**P < 0.01$; $*P < 0.05$. (D) Soft agar colony formation assay. Data were presented as the mean ± SEM from at least three separate experiments and each experiment was preformed in triplicate. $**P < 0.01$; $*P < 0.05$. (E) Effects of NDRG2 on tumorigenicity of EC-7906 and EC 109 cells in nude mice were evaluated by measuring the sizes of excised tumors at the time of death. Tumors were verified as esophageal cancer by H&E staining.$*P < 0.05$.

untreated and transfected with pcDNA3.1-NC groups in both EC9706 and EC109 cells. However, the apoptotic rate of EC9706 and EC109 cells transfected with pcDNA3.1-NDRG2 were significantly increased compared with untreated and pcDNA3.1-NC groups (Fig. 4A1,2). Cyclin D1 and Bcl-2 play a key role in cell apoptosis. We then investigated the expression of cyclin D1 and Bcl-2 by western blotting. Our results indicated that the expression level of cyclin D1 and Bcl-2 were down-regulated by pcDNA3.1-NDRG2 transfection in EC9706 cells (Fig. 4B), which indicated that the activated NDRG2 signaling pathway may induce apoptosis in EC9706 cells through down-regulation of cyclin D1 and Bcl-2 proteins.

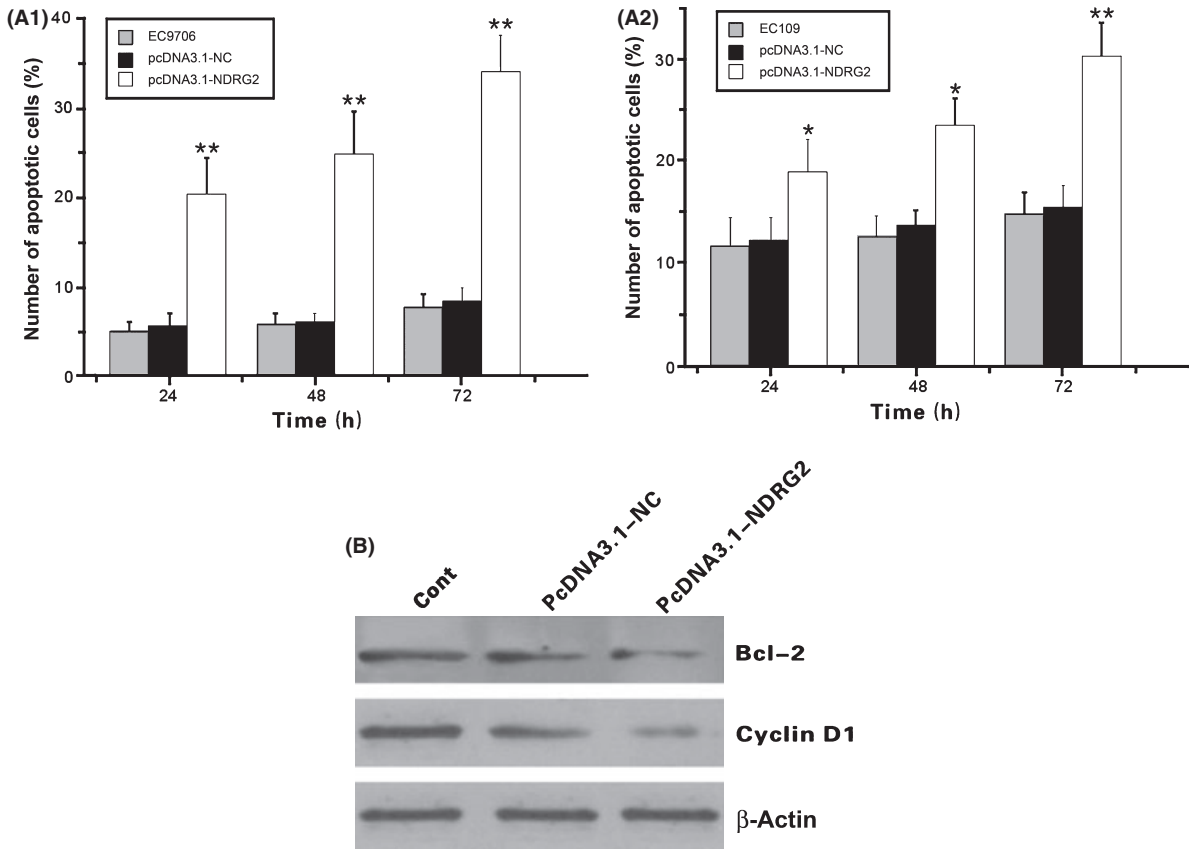

Fig. 4. Activation of N-Myc downstream-regulated gene 2 (NDRG2) signaling induces cell apoptosis in EC9706 and EC109 cells. (A1) EC9706 cells untreated and transfected with pcDNA3.1 and pcDNA3.1-NDRG2 were seeded in six-well plates containing 10% FBS-RPMI-1640 and harvested at 24, 48, and 72 h, respectively, followed by apoptosis assay using the Annexin V-FITC apoptosis detection kit. (A2) EC109 cells untreated and transfected with pcDNA3.1 and pcDNA3.1-NDRG2 were seeded in six-well plates containing 10% FBS-RPMI-1640 and harvested at 24, 48, and 72 h, respectively, followed by apoptosis assay using the Annexin V-FITC apoptosis detection kit. $^{*}P < 0.05$; $^{**}P < 0.01$. (B) Effects of NDRG2 on apoptotic proteins. The expression of Bcl-2 and cyclin D1 was significantly reduced in EC9706 cells with overexpression of NDRG2 and no profound changes were observed in the expression of Bcl-2 and cyclin D1 in cells without NDRG2 transfection. β-Actin was used as an internal control.

Discussion

NDRG2, together with NDRG1, NDRG3, and NDRG4, constitute the NDRG gene family. Many data have demonstrated the importance of this gene family in cell proliferation and differentiation. Significant attention has been paid to the NDRG gene family because of its potential as a tumor suppressor.[8,10,11]

Several research groups have investigated and confirmed that NDRG2 was differently expressed between tumor and normal tissues. Lusis *et al.* found that NDRG2 expression was consistently down-regulated in grade III meningioma at both the transcript and protein levels.[27] Choi *et al.* demonstrated that only two gastric cancer cell lines, SNU-16 and SNU-620, expressed NDRG2 among seven gastric cancer and two noncancer cell lines. The survival rate of NDRG2-negative patients was lower than that of NDRG2-positive patients.[17] Furuta *et al.* showed that NDRG2 was expressed at a lower level in oral squamous-cell carcinoma than in normal tissues, and up-regulation of NDRG2 expression in an HSC-3/OSCC cell line significantly inhibited cell proliferation and decreased colony formation ability on soft agar.[28] The same trend has been obtained in colon, liver, pancreatic, breast, and thyroid cancer by our group.[15,23,29,30]

The present study confirmed that the *NDRG2* gene was a differentially expressed gene in ESCC, and the expression level of NDRG2 was closely related with prognosis of patients with ESCC. Up-regulation of NDRG2 expression in EC109 and EC9706 cell lines demonstrated that NDRG2 might be involved in the regulation of ESCC cell proliferation and apoptosis. NDRG2 could suppress the proliferation of ESCC cells via down-regulating the inhibitors of apoptosis (cyclin D1 and Bcl-2).

NDRG2 was a MYC-repressed gene, and its expression was controlled by MYC and other factors. Wang *et al.* found that NDRG2 is a new hypoxia-inducible factor 1 (HIF-1) target gene necessary for hypoxia-induced apoptosis in A549 cells.[31] Svensson *et al.* demonstrated that the Wilms' tumor gene 1 (WT1) induces expression of NDRG2 directly or indirectly.[32] Nichols showed that protein of NDRG2 was regulated by adrenal steroids and antidepressants.[13]

Zhang *et al.* provided evidence that the expression of human NDRG2 is down-regulated by Myc via transcriptional repression.[9] The ectopic expression of C-MYC dramatically reduces the cellular NDRG2 protein and mRNA levels. Furthermore, this confirmed the core promoter region of NDRG2 necessary for MYC repression on NDRG2 transcription and verified the interaction of MYC with the core promoter region both *in vitro* and *in vivo*. Shi *et al.* also obtained the same trend for NDRG2 and C-MYC in colon cancer.[15] Our current results regarding NDRG2 and C-MYC obtained from 154 cases of human ESCC were in agreement with previous reports. While NDRG2 expression was found to be reduced in ESCC, as compared with

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adjacent and normal colorectal tissue from the same individuals, a reversed expression pattern was observed for C-MYC, which was abundant in carcinoma tissues and suppressed in normal tissues. These results suggest that the transcriptional repression of human NDRG2 by C-MYC might participate in the carcinogenesis of ESCC.

Taken together, our results revealed that *NDRG2* and *C-MYC* are differentially expressed genes in ESCC, and NDRG2 might be as a prognostic biomarker. Our data do provide evidence that NDRG2 expression correlates with the proliferation and apoptosis of ESCC cells. However, further studies are required to classify the biological functions of NDRG2 and elucidate the specific roles of NDRG2 in the invasion and metastasis of ESCC.

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