NDRG1/Cap43/Drg-1 may Predict Tumor Angiogenesis and Poor Outcome in Patients with Lung Cancer

Koichi Azuma, MD, PhD,* Akihiko Kawahara, PhD,† Satoshi Hattori, PhD,‡
Tomoki Taira, PhD,† Junji Tsurutani, MD, PhD,§ Kosuke Watari, PhD,¶ Tomohiro Shibata, PhD,||
Yuichi Murakami, PhD,|| Shinzo Takamori, MD, PhD,¶ Mayumi Ono, PhD,¶ Hiroto Izumi, MD, PhD,§
Masayoshi Kage, MD, PhD,† Takashi Yanagawa, PhD,‡ Kazuhiko Nakagawa, MD, PhD,§
Tomoaki Hoshino, MD, PhD,* and Michihiko Kuwano, MD, PhD**

Objective: Expression of N-myc downstream-regulated gene 1 (NDRG1)/Cap43 is a prognostic indicator of human malignancies according to the tumor type in which it occurs. We investigated how NDRG1/Cap43 could affect tumor growth and angiogenesis in non–small-cell lung cancer (NSCLC) in vivo using an animal experimental model, and also how it could affect tumor angiogenesis and prognosis in NSCLC patients.

Methods and Results: Knockdown of NDRG1/Cap43 in lung cancer cells using a specific small interfering RNA resulted in growth rates in culture that were similar to those of counterpart control cells, but decreased tumor growth rates in vivo markedly. Stable NDRG1/Cap43 knockdown did not induce consistent changes in the expression of Epidermal growth factor receptor (EGFR) family proteins and c-Met in two human lung cancer cell lines in vitro. However, cell lines with NDRG1/Cap43 knockdown showed markedly decreased production of the potent angiogenic factors vascular endothelial growth factor-A and interleukin-8. Cells with knockdown of NDRG1/Cap43 showed marked reduction of tumor-induced angiogenesis. Using immunohistochemistry, we examined 182 surgically resected specimens of NSCLC for expression of NDRG1/Cap43 and tumor angiogenesis. High microvessel density in the tumor was significantly associated with nuclear positivity for NDRG1/Cap43 in both adenocarcinoma (p = 0.003) and squamous cell carcinoma (p = 0.041). For both adenocarcinoma (p = 0.031) and squamous cell carcinoma (p = 0.034), the survival curve of patients negative for nuclear NDRG1/Cap43 expression differed significantly from that of patients who were positive.

Conclusion: Therefore, the expression of NDRG1/Cap43 may be predictive of tumor angiogenesis and poor prognosis in NSCLC.

Key Words: NDRG1/Cap43, Non–small-cell lung cancer, Epidermal growth factor receptor, Immunohistochemistry, Angiogenesis.

N-myc downstream-regulated gene 1 (NDRG1)/Cap43 has been identified as a nickel- and calcium–inducible gene, and is identical to both homocysteine-inducible gene reduced in tumor cells (RTP/rit42), and differentiation-related gene-1(Drg-1). The expression of NDRG1/Cap43 is markedly influenced by various stimuli, including oxidative stress, metal ions, hypoxia, phorbol esters, vitamins A and D, steroids, homocysteine, and tunicamycin, and oncogenes (N-myc and C-Myc) and tumor suppressor genes (p53 and von Hippel-Lindau). NDRG1/Cap43 is expressed in various organs, including the prostate, ovary, colon, and kidney, and its expression changes dynamically during postnatal development in the kidney, brain, liver, and nerves.

Our previous study demonstrated that overexpression of NDRG1/Cap43 markedly suppressed tumor angiogenesis and growth of human pancreatic cancer, and also that the level of NDRG1/Cap43 expression was significantly and inversely correlated with longer overall survival and tumor microvascular density (MVD) in patients with pancreatic cancer. In patients with breast and prostate cancer and patients with neuroblastoma, a low level of NDRG1/Cap43 expression has reportedly been correlated with poor prognosis. In contrast, high NDRG1/Cap43 expression is related to poor prognosis and angiogenesis in cervical adenocarcinoma and gastric cancer. A related study has also shown that high NDRG1/Cap43 expression is correlated with tumor differentiation, vascular invasion, and poor prognosis in patients with hepatocellular carcinoma. Thus it seems that whether high NDRG1/Cap43 expression suppresses tumor growth and angiogenesis depends upon the type of human malignancy.
Lung cancer continues to be the leading cause of cancer deaths worldwide. Non–small-cell lung cancer (NSCLC) is the most common type of lung cancer, accounting for approximately 80% of cases. More than half of affected patients already have metastasis at the time of diagnosis, and chemotherapy is the most effective treatment. Although many clinical trials of platinum-based chemotherapy in combination with various drugs have been conducted, the median survival time of NSCLC patients remains poor. The overall 5-year survival is approximately 15%, and has improved only marginally over the last few decades despite progress in new anticancer therapeutic agents. Recent progress in novel therapeutics, including molecular targeting drugs, has improved therapeutic efficacy in some NSCLC patients. Furthermore, development of new agents for cancer therapy, including vascular endothelial growth factor (VEGF)-A–targeted drugs, is expected to bring further benefits to NSCLC patients. In the present study, we investigated whether NDRG1/Cap43 expression in lung cancer cells could affect the growth and angiogenesis of tumor xenografts in mice, and also whether NDRG1/Cap43 expression could affect the outcome of patients with NSCLC.

MATERIALS AND METHODS

Cells, Cell Culture, and Immunoblotting

Human lung cancer cell lines A549, PC9, 11_18, LK87, LC-1 (adenocarcinoma), QG56, LC-Sq-1, and RERF-LC-AI (squamous cell carcinoma) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and incubated in a humidified atmosphere of 5% CO2 at 37°C. Anti-NDRG1/Cap43 antibodies were produced in our laboratory.7,26 PC9 and QG56 cells were kindly provided by Dr. Yukito Ichinose (Kyushu Cancer Center, Fukuoka, Japan) and LK87, LC-1, LC-Sq-1, and RERF-LC-AI cells were kindly provided by Kyogyo Itho (Kurume University, Fukuoka, Japan). The rabbit polyclonal antibody against NDRG1/Cap43 was used as described previously.13 Anti-EGFR antibody was obtained from Cell Signaling Technology (Beverly, MA), anti-HER2 was purchased from Upstate, Inc. (Lake Placid, NY), anti-HER3 was obtained from IBL Laboratories (Gunma, Japan), and anti-beta-actin was purchased from Sigma Aldrich (St. Louis, MO).

Small Interfering RNA Transfection and Immunoblotting

Small interfering RNA (siRNA) corresponding to a nucleotide sequence of NDRG1/Cap43 (5′-AACTGCCGTCAGCCCTTGTGGAGAAATTCA AGAGATTCCGCACAAGGTCTCAGTGTGCGGAATTCA AGATCTTTTCCAAAACGTCGAACCCCTTGTCGGG AATCCTTT 3′) and 5′-AGCTTTTCCAAAACGTCGAACCCCTTGTCGGG AATCCTTT 3′, respectively. The amplified Cap43 complementary DNA was then ligated into the pcDNA3_GFP_U6siRNA 1.0 vector (Invitrogen, Carlsbad, CA) (pcDNA3-Cap43). Cells were transfected with pcDNA3-Cap43 or pcDNA3-Mock using LipofectAMINE 2000 (Invitrogen) in accordance with the manufacturer’s protocol. Stable transfected clones were established using G418 selection.

Determination of VEGF and Interleukin-8 by ELISA

The concentrations of VEGF and interleukin-8 (IL-8) in the conditioned medium and tissue lysates were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits.13 Cells were plated in 24-well dishes in medium containing 10% FBS. When the cells reached subconfluence, the medium was replaced with Dulbecco’s Modified Eagle Medium containing 2% FBS, and then the cells were incubated for another 24 hours. Results were normalized for the number of cells and expressed as picograms of growth factor/105 cells/24 hours. The concentrations of VEGF and IL-8 in the lysate supernatants were measured using an ELISA kit in accordance with the manufacturer’s protocols.

Animals

All animal studies were done in accordance with the Recommendations for Handling of Laboratory Animals for Biomedical Research compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animal Experiments, Kinki University, Higashiosaka, Osaka, Japan. The ethical procedures followed met the requirements of the United Kingdom Coordinating Committee on Cancer Research guidelines.27 Male athymic nude mice were exposed to a 12-hour light, 12-hour dark cycle and provided with food and water ad libitum in a barrier facility.

Tumor cells were implanted subcutaneously into the right hind leg of individual 6-week-old female athymic nude mice (BALB/c nu/nu; CLEA Japan). Tumor volume was determined from caliper measurements of tumor length (L) and width (W) according to the formula L x W²/2. Tumor size was measured twice per week. Animals were observed for signs of tumor growth, activity, feeding, and pain in accordance with the guidelines of the Harvard Medical Area Standing Committee on Animals.
Nude Mouse Xenograft Models and Determination of MVD

Cells were suspended in sterile phosphate-buffered saline at a concentration of 10⁴ cells/ml, and 100 AL were injected s.c. into the right flank of individual nude mice. Tumor size was measured using calipers across the largest diameter and perpendicular to it to calculate the tumor area. Intratumoral microvessels were detected using a rat anti-mouse CD34 antibody as described previously. Tumors in nude mice were removed, snap-frozen in optimum cutting temperature compound (Sakura Fine Technical, Japan), and 6-Am sections were cut, air-dried, and fixed in cold acetone for 10 minutes. The sections were blocked with 3% bovine serum albumin and labeled at room temperature with rat anti-mouse CD34 for 1 hour, followed by biotinylated goat anti-rat IgG for 20 minutes. Intratumoral microvessels in human specimens were detected using a monoclonal antibody against CD34 antigen (monoclonal mouse anti-human CD34 antibody; Nichirei, Tokyo, Japan). In all samples, the mean number of microvessels was calculated from four vascular hotspots, and assessed as the MVD for each case. Only CD34 staining in the tumor area was reviewed, and any endothelial cell cluster consisting of two or more cells was considered to be a single, countable microvessel. All counts were done by three independent observers without any knowledge of the corresponding clinicopathologic data.

Mouse Dorsal Air Sac Assay

A549/mock5 or A549/Cap9 cells (1 × 10⁶ cells) were injected into a chamber that consisted of a ring (Micro Industries Co., Ltd., Tokyo, Japan), and implanted into an air sac as described previously. On day 5, the chambers were removed, and photographs of these sites were assessed, followed by determination of newly formed vessels.

Patients and Tumor Samples

We examined 182 patients with primary NSLSC whose tumors had been completely removed surgically at the Department of Surgery, Kurume University, Kurume, Japan, between 1997 and 2005. Among the 182 patients, 115 were diagnosed histologically as having adenocarcinoma, and the other 67 were diagnosed as having squamous cell carcinoma. The age of the patients with NSCLC ranged from 41 to 82 years (median, 64 years), 117 were men and, 65 were women. None of the patients had received neoadjuvant or adjuvant chemotherapy. The median follow-up period was 1511.5 days (range, 159 to 3801 d). The present study conforms to the provisions of the Declaration of Helsinki, and was approved by the Institutional Review Board of Kurume University.

Immunohistochemistry

Paraffin-embedded tissue samples were cut at a thickness of 4 μm and examined on coated glass slides, after labeling with antibodies directed against NDRG1/Cap43 and CD34. For NDRG1/Cap43, the BenchMark XT was used (Ventana Automated Systems, Inc., Tucson, AZ). This automated system employs the streptavidin-biotin complex method with 3,3’ diaminobenzidine as the chromogen (Ventana iVIEW DAB Detection Kit). Antigen retrieval of NDRG1/Cap43 was done by heat treatment in a CC1 (Ventana). CD34 antigen retrieval was done by treatment with Proteinase K for 5 minutes. Each slide was incubated overnight with the antibody at 4°C. For staining detection, the ChemMate ENVISION method (DakoCytomation, Glostrup, Denmark) was used with 3,3’ diaminobenzidine as the chromogen.

Evaluation of Immunohistochemistry

The expression of NDRG1/Cap43 protein in the cytoplasm and nucleus was investigated in detail. Cytoplasmic NDRG1/Cap43 expression was classified into three categories: score 0, no staining at all, or faint/barely perceptible partial membrane expression in less than 10% of cancer cells; score 1+, weak-to-moderate expression on the entire membrane in more than 10% of the cancer cells; score 2+, strong expression on the entire membrane in more than 10% of cancer cells. Nuclear NDRG1/Cap43 expression was classified into three categories: score 0, no staining at all; score 1+, nuclear expression in less than 10% of the cancer cells; score 2+, nuclear expression in more than 0% of the cancer cells. The extent of immunohistochemistry (IHC) for NDRG1/Cap43 was defined as follows: a score of 2+ was regarded as positive, and a score of 0 or 1 as negative. All IHC studies were evaluated by two IHC-experienced reviewers who were blind to the conditions of the patients (M. Kage and A.K).

MVD Analysis

MVD analysis was performed to measure the area of CD34 expression in all cases, using the Win ROOF (version 5.7, Mitani Corporation, Osaka, Japan) software package. Images of microvessels were selected for clarity in each of 5 high-power fields from each immunohistochemically stained specimen, using a CCD digital camera (DXM1200; Nikon, Melville, NY). The MVD was measured, and then averaged.

Statistical Analysis

The mean profile of A549/Mock-5 and that of A549/Cap9 were graphically presented over time. To determine whether the means were significantly different at given time points after injection, we analyzed the average tumor volumes determined after 40 days (days 41, 48, and 56) to stabilize any variation in observations. After the tumor volumes of both A549/Mock-5 tumors and A549/Cap9 were obtained from each mouse, the average tumor volumes after day 40 were compared using the paired t test. Distributions of averaged MVD were demonstrated with box-plots and compared between NDRG1/Cap43-positive and -negative patients using the Wilcoxon rank-sum test. Overall survival was defined as the time from surgery until the date of death resulting from any cause. The relationships between NDRG1/Cap43 expression and overall survival were examined by the Kaplan-Meier method and the log-rank test. To evaluate the effect of NDRG1/Cap43 on overall survival with adjustment for possible confounding factors, Cox regression analysis was performed. Differences were regarded as significant at p < 0.05 unless otherwise indicated. Statistical analysis was performed.
on the basis of histological type using SAS version 9.1 (SAS Institute Inc., Cary, NC) and R version 2.7.0.

RESULTS
Effects of NDRG1/Cap43 Knockdown on Expression of EGFR Family Proteins and Cell Proliferation in Human Lung Cancer Cells Are Not Consistent

To examine whether there are any correlations of expression between NDRG1/Cap43 and various growth factor receptors, we examined the expression levels of NDRG1/Cap43 in relation to those of EGFR, HER2, HER3, and c-Met in eight NSCLC cell lines. Among these cell lines, LK87, QG56, and RERF-LC-AI showed relatively higher expression of NDRG1/Cap43 than A549, whereas PC9, LC1, 11_18, and LC-Sq1 showed relatively lower (about 30% or less) NDRG1/Cap43 expression. The levels of HER2, HER3, and c-Met expression were decreased to various extents in LC1, 11_18, and LC-Sq1 when the expression of NDRG1/Cap43 was relatively lower. By contrast, the expression of EGFR seemed to be relatively higher in PC9 and LC-Sq1 (Fig. 1A). However, an overall survey using Western blot analysis revealed no apparent correlation between the level of NDRG1/Cap43 expression and that of various growth factor receptors.

We next examined the effect of NDRG1 knockdown using cognate siRNA on the expression of EGFR family proteins and c-Met. Transient knockdown by transfection of A549 cells with NDRG1 siRNA decreased the expression of HER2 and HER3 by about 30% to 50%, but changed the expression of EGFR and c-Met only negligibly (Fig. 1B and C). In QG56 cells, NDRG1 knockdown markedly reduced the expression of EGFR, but did not affect the expression of HER2, HER3, and c-Met (Fig. 1B and C). Thus, in these two lung cancer cell lines, transient transfection with NDRG1/Cap43 siRNA did not induce consistent changes in the expression of growth factor receptors.

We next established stable NDRG1/Cap43-knockdown lung cancer cell lines by transfection of NDRG1 shRNA into both A549 and QG56. The resulting cell lines (A549/Sic-9 and A549/Sic-11; QG56/Sic-2 and QG56/Sic-12) showed cell proliferation rates similar to those of their respective parental counterparts (Fig. 2A). A549/Sic-9 showed decreased HER3 expression relative to A549/mock5, but no apparent changes in the expression of other growth factor receptors (Fig. 2B). By contrast, in another NDRG1/Cap43-knockdown cell line, the A549/Sic-11, expression of EGFR, but not that of other growth factor receptors, was specifically downregulated (Fig. 2B). Conversely, relative to QG56/Mock2, the expression of HER3 in QG56/Sic-12 was increased, whereas that in QG56/Sic-2 was similar. There were no apparent changes in the

FIGURE 1. Lack of correlation between NDRG1/Cap43 expression level and expression of EGFR, HER2, HER3, and c-MET in human lung cancer. A, Expression of NDRG1/Cap43 in seven cell lines was determined by Western blot analysis in a human lung cancer cell line panel. B, A549 and QG56 cells were treated with 25, 50, and 100 nmol/l NDRG1 siRNA for 72 hours, and the effects of NDRG1 knockdown on expression of EGFR, HER2, HER3, and c-MET were evaluated by Western blotting. NDRG1, N-myc downstream-regulated gene 1; siRNA, Small interfering RNA.
expression of other growth factors between QG56/Sic-2 and QG56/Sic-12 relative to those in QG56/Mock2 (Fig. 2B). Two NDRG1/Cap43 knockdown cell lines of A549 showed differential effects on the expression of growth factor receptors including EGFR and HER3, suggesting the functional diversity of NDRG1/Cap43 in lung cancer cells. However, there was no change in the cell proliferation rate between NDRG1/Cap43 knockdowned cell lines and their parental counterpart. Furthermore, in A549 and QG56, there were no apparent changes in the expression of Akt, phospho-Akt (pAkt), extracellular signal-regulated kinase (ERK), and phosphor-ERK (pERK) between NDRG1/Cap43 knockdowned cell lines and mock cell lines. These data suggest that A549 and QG56 cells show no consistent changes in the expression of growth factor receptors resulting from NDRG1 knockdown, and also that altered expression of growth factor receptor by NDRG1 knockdown does not affect cell proliferation.

NDRG1/Cap43 Knockdown Induces Downregulation of Angiogenic Factors and Tumor Angiogenesis

Our previous studies revealed that overexpression of NDRG1/Cap43 suppresses tumor angiogenesis in pancreatic cancer, suggesting that NDRG1/Cap43 may be an angiogenesis-suppressor gene.13 However, in other human malignancies such as gastric cancer14 and cervical cancer,17 the NDRG1 overexpression is inversely correlated with tumor angiogenesis. Therefore, NDRG1 may promote or suppress tumor angiogenesis according to tumor type. Using the enzyme-linked immunosorbent assay, we compared the expression levels of two potent angiogenic factors, VEGF-A and IL-8/CXCL8, between NDRG1-knockdown cell lines and their parental counterparts (Fig. 3). All the NDRG1/Cap43-knockdown cell lines derived from A549 and QG56 showed significantly (p < 0.05) reduced expression of VEGF-A and IL-8/CXCL8.

We then investigated whether NDRG1/Cap43 knockdown using a xenograft assay system could modulate tumor growth in mice. The tumor growth rate of A549/Sic-9 cells in vivo was markedly reduced in comparison with the corresponding mock-transfected line, A549/Mock-5 (Fig. 4A). The average tumor volume at day 40 differed significantly between A549/Sic-9 and A549/Mock-9 (p = 0.0162). However, no in vivo tumor growth was evident for QG56/Sic-2, QG56/Sic-12, and QG56/Mock2 cells (data not shown). We further compared tumor angiogenesis between A549/Sic-9 tumors and A549/Mock9 tumors by IHC with anti-CD34 antibody (Fig. 4B). A549/Sic-9 tumors showed less development of neovessels than A549/Mock-5 tumors (Fig. 4B). Quantitative analysis revealed a significant decrease in the number of MVD in A549/Sic-9 tumors by more than 50% relative to the control counterpart tumors (Fig. 4C).

We then investigated whether NDRG1 knockdown suppressed angiogenesis by cancer cells in vivo using the dorsal air sac assay. Implantated A549/Mock5 cells developed thin
and curled microvessels with tiny bleeding spots, in addition to the preexisting vessels (Fig. 4D), consistent with our previous study.24 By contrast, A549/Sic9 showed reduced development of newly formed vessels. Quantitative analysis revealed a reduction of angiogenesis by about 60% in mice harboring the A549/Sic-9, as opposed to A549/Mock5, xenografts (Fig.4E).

Association of NDRG1 Expression with Angiogenesis, Clinicopathologic Characteristics, and Prognosis in NSCLC Patients

Using IHC analysis we examined whether the NDRG1/Cap43 expression was associated with clinicopathologic features in tissue samples from 182 patients with NSCLC. The tumor histology was classified as adenocarcinoma in 115 patients and squamous cell carcinoma in 67. We scored the levels of NDRG1/Cap43 expression in the cytoplasm and nucleus, respectively, of cancer cells. Figure 5A and B shows representative examples of IHC staining for NDRG1/Cap43 expression in the cytoplasm and nucleus of lung adenocarcinoma and squamous cell carcinoma cells.

Nuclear NDRG1/Cap43 expression was positive in 102 patients and negative in 80, whereas cytoplasmic NDRG1/Cap43 expression was positive in 81 patients and negative in 101. In adenocarcinoma, NDRG1/Cap43 nuclear expression was positive in 44 patients and negative in 71, and cytoplasmic expression was positive in 64 and negative in 51. In squamous cell carcinoma, nuclear NDRG1/Cap43 expression was positive in 58 patients and negative in 9, and cytoplasmic NDRG1/Cap43 expression was positive in 37 patients and negative in 30.

Clinical and pathological characteristics of the 182 patients analyzed in this study at diagnosis are summarized in Table 1. Results of Fisher’s exact test for association between molecular markers and NDRG1/Cap43 are shown in Table 1. In adenocarcinoma, there was a significant correlation between age and cytoplasmic NDRG1/Cap43 expression (p=0.0388), whereas there was no correlation between NDRG1 and sex, p-stage, and smoking status (Table 1). In squamous cell carcinoma, none of the above factors were correlated with nuclear and cytoplasmic expression of NDRG1/Cap43. Cytoplasmic expression of NDRG1/Cap43 was significantly correlated with age in adenocarcinoma (p=0.0388), but not in squamous cell carcinoma (p=0.796). In adenocarcinoma, the ratio of the proportion of the highly expressed in the subgroup of age 65 relative to that of age 64 was estimated at 0.62 (95% confidence interval [CI] 0.41–0.97), indicating that NDRG1/Cap43 in cytoplasm is likely to be less expressed in older patients. The Kaplan-Meier plots for overall survival in patients with high and low expression of nuclear NDRG1/Cap43 are shown for adenocarcinoma and squamous cell carcinoma in the left and right panels of Figure 5C, respectively. The survival curves differed significantly according to the expression of nuclear NDRG1/Cap43 in both adenocarcinoma (p=0.031; hazard ratio [HR]=1.70, 95%CI 1.05–2.78) and squamous cell carcinoma (p=0.034; HR=4.16, 95%CI 1.00–17.40). Conversely, the cytoplasmic expression of NDRG1/Cap43 was not associated with overall survival in adenocarcinoma (p=0.687; HR=1.13, 95%CI 0.69–1.84) or squamous cell carcinoma (p=0.954; HR=1.02, 95%CI 0.55–1.90). To evaluate the effect of nuclear NDRG1/Cap43 on overall survival while adjusting for possible confounding factors, Cox regression analysis was performed for adenocarcinoma. No such analysis was performed for squamous cell carcinoma because the number of patients showing high nuclear expression of NDRG1/Cap43 was small. Estimated HRs are presented in Table 2. MVD, which is associated with expression of nuclear NDRG1/Cap43, was not strongly predictive of overall survival (p=0.692) (Fig. 6B). Contrary to this, even after adjustment for MVD and age, patients with high nuclear expression of NDRG1/
Cap43 had significantly shorter overall survival than those with low expression ($p=0.0298$; HR=1.76, 95% CI 1.06–2.92) (Table 2).

**Association Between NDRG1/Cap43 Expression and Tumor Angiogenesis in NSCLC**

Finally, we examined whether NDRG1/Cap43 affected lung cancer angiogenesis in human patients. Tumor angiogenesis was evaluated by IHC analysis of clinical tumor samples using anti-CD34 antibody. Representative IHC images showing high and low NDRG1/Cap43 expression are presented in Figure 6A.

Figure 6C shows the distributions of MVD using box-plots according to the expression of nuclear NDRG1/Cap43. MVD was higher in adenocarcinoma than in squamous cell carcinoma. In patients with adenocarcinoma, the median MVD in patients with high expression of nuclear NDRG1/Cap43 was 4370 (q1-q3:2924–7192), whereas that in patients with low expression was 2793 (q1-q3:2024–4216). In squamous cell carcinoma, the corresponding values in patients showing high and low expression were 1146 (q1-q3:698–1505) and 731 (q1-q3:306–1035), respectively. In both adenocarcinoma ($p=0.003$) and squamous cell carcinoma ($p=0.041$), the difference in the median values was significantly higher in patients with high expression of nuclear NDRG1/Cap43 than in patients with low expression (Fig. 6C). Expression of NDRG1/Cap43 in the cytoplasm was not associated with MVD in either adenocarcinoma or squamous cell carcinoma (data not shown).
DISCUSSION

IHC for NDRG1/Cap43 expression showed that NDRG1 was expressed only negligibly in normal lung tissue, but was often expressed in either the nucleus or cytoplasm of cancer cells in both adenocarcinoma and squamous cell carcinoma (Fig. 5A, B, C). Quantitative analysis showed that among the various factors examined, the expression of NDRG1 in the nucleus was significantly correlated with survival of NSCLC patients overall ($p=0.0298$), and also that of patients with adenocarcinoma ($p=0.031$) and squamous cell carcinoma ($0.034$). NDRG1 expression in cancer cells is a predictive marker of good outcome in patients with cancers of the prostate, breast, esophagus, colon, and pancreas, as well as neuroblastoma.\textsuperscript{13-16,29,30} By contrast, NDRG1 expression is a predictive marker of poor outcome in patients with liver, cervical, and gastric cancer.\textsuperscript{17-19} Our present study indicated

FIGURE 5. Expression of NDRG1/Cap43 in human lung cancer specimens analyzed by immunohistochemistry (IHC). A and B, Nuclear and cytoplasmic expression of NDRG1/Cap43 can be seen in the tumor cells of adenocarcinoma (A) and squamous cell carcinoma (B). C, Kaplan-Meier analysis of overall survival in relation to NDRG1/Cap43 expression levels for adenocarcinoma (Fig. 5C left) and squamous cell carcinoma (Fig. 5C right). NDRG1, N-myc downstream-regulated gene 1.
that NDRG1 expression was predictive of poor outcome in patients with NSCLC. Collectively, the data suggest that the ability of NDRG1 expression to predict a good or poor outcome depends upon the type of cancer. It still remains unclear why NDRG1/Cap43 could have a double-edged influence on cancer progression. NDRG1/Cap43 was originally isolated as a gene controlled by N-Myc and/or c-Myc. In some tumor types, NDRG1/Cap43 is strictly controlled by the Myc oncogene, whereas in others, it is not. The presence or absence of Myc-driven control of NDRG1/Cap43 seems to be different among tumor types. In contrast, in pancreas cancer cells, we have previously reported that NDRG1 is predictive of good prognosis, and also that NDRG1 suppresses tumor angiogenesis and growth through its inhibitory effect on the NF-κB pathway. Together, which signaling pathway including transcriptional factors could be activated or inactivated under the control of NDRG1 is also expected to be responsible for the double-edged function of NDRG1.

It has been shown that knockdown of NDRG1 does not alter the growth rates of human pancreas, prostate or colon cancer cells. Consistent with these findings, we showed here that the A549 and QG56 cell lines with NDRG1 knockdown had growth rates similar to those of their parental counterparts in culture. However, tumor growth in vivo was markedly decreased by NDRG1 knockdown in A549 cells, in comparison to the high growth rate of their parental counterpart cells. Thus NDRG1 downregulation seemed to specifically inhibit tumor growth in vivo but not during cell proliferation in vitro, suggesting a suppressive role of NDRG1/Cap43 against the development of neovessel tumor stroma by NSCLC cells. NDRG1 knockdown also suppressed production of the potent angiogenic factors VEGF and IL-8 by both types of human lung cancer cells, suggesting the involvement of reduced expression of such angiogenic factors in the poor angiogenesis resulting from NDRG1 knockdown. Conversely, NDRG1 specifically suppressed the tumor angiogenesis and growth of pancreatic cancer cells, suggesting that NDRG1 is a putative angiogenesis-suppressor gene. In these previous studies, the expression of VEGF, IL-8 and other angiogenic CXC chemokines was markedly reduced in pancreatic cancer cells as a result of NDRG1 overexpression. Although it remains to be clarified how NDRG1 promotes or suppresses angiogenesis by cancer cells, the role of NDRG1 as an angiogenesis promoter or suppressor may depend upon tumor species or cancer cell type.

Our previous study demonstrated for the first time that NDRG1/Cap43 overexpression markedly decreased tumor angiogenesis and tumor growth in mice bearing human pancreatic cancer xenografts. NDRG1 overexpression markedly reduced the expression of angiogenic factors such as VEGF, IL-8, and matrix metalloproteinase-9 in cultured pancreatic cancer cells. Consistent with in vitro and in vivo experimental results, IHC analysis also demonstrated an inverse correlation between NDRG1 expression and MVD in clinical samples from pancreatic cancer patients. However, our present study showed that NDRG1 exerted opposite effects on tumor angiogenesis

### TABLE 1. Association of Nuclear and Cytoplasmic NDRG1/Cap43 Expression with Pathological Stage and Other Characteristics

<table>
<thead>
<tr>
<th>Histological type</th>
<th>Characteristics</th>
<th>Number</th>
<th>NDRG1/Cap43 (nuclear)</th>
<th>NDRG1/Cap43 (cyto)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p value</td>
<td></td>
</tr>
<tr>
<td>Squamous cell carcinoma N=67</td>
<td>Age −64</td>
<td>21</td>
<td>4 (19.00%)</td>
<td>17 (81.00%)</td>
</tr>
<tr>
<td></td>
<td>65− 46</td>
<td>5</td>
<td>10 (10.90%)</td>
<td>41 (89.10%)</td>
</tr>
<tr>
<td></td>
<td>Sex F</td>
<td>5</td>
<td>1 (20.00%)</td>
<td>4 (80.00%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M 62</td>
<td>8</td>
<td>12 (9.80%)</td>
<td>54 (87.10%)</td>
</tr>
<tr>
<td></td>
<td>pStage I</td>
<td>31</td>
<td>12 (9.90%)</td>
<td>27 (87.10%)</td>
</tr>
<tr>
<td></td>
<td>II 16</td>
<td>3</td>
<td>18 (8.00%)</td>
<td>13 (82.00%)</td>
</tr>
<tr>
<td></td>
<td>III 20</td>
<td>2</td>
<td>10 (10.00%)</td>
<td>18 (90.00%)</td>
</tr>
<tr>
<td></td>
<td>Smoker 63</td>
<td>4</td>
<td>25.00%</td>
<td>75.00%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### TABLE 2. Multivariate Analysis of Overall Survival

<table>
<thead>
<tr>
<th></th>
<th>p Value</th>
<th>HR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDRG1/Cap43 (nucleus)</td>
<td>0.0298</td>
<td>1.757</td>
<td>1.057−2.922</td>
</tr>
<tr>
<td>Tumor angiogenesis (MVD)</td>
<td>0.6916</td>
<td>0.929</td>
<td>0.647−1.335</td>
</tr>
<tr>
<td>Age</td>
<td>0.6026</td>
<td>0.993</td>
<td>0.969−1.018</td>
</tr>
</tbody>
</table>

HR, hazard ratio; CI, confidence interval; NDRG1, N-myc downstream-regulated gene 1; MVD, microvascular density.
in the dorsal air sac assay and in clinical samples of NSCLC. Furthermore, in NSCLC, NDRG1/Cap43 was more predictive of higher tumor angiogenesis than of lower. A related study by Nishio et al. found that cervical cancers with higher NDRG1 expression showed higher MVD, suggesting that NDRG1 may promote angiogenesis in cervical cancer. Consistent with the positive correlation of NDRG1 with tumor angiogenesis, NDRG1 expression also showed a significant correlation with tumor angiogenesis or poor prognosis in patients with gastric cancer. Together, the data suggest that NDRG1 expression is predictive of tumor angiogenesis in lung cancer, as is the case in cervical and gastric cancer.

In conclusion, we have shown that NDRG1 is differentially expressed in the cytoplasm and nucleus of NSCLC cells, but exhibits a close mutual association, suggesting that expression of NDRG1 in the nucleus parallels that in the cytoplasm. Of the many biomarkers that are already known to predict poor prognosis of lung cancer, NDRG1 will be potentially applicable as a novel biomarker of prognosis in its close association with tumor angiogenesis, and might be useful for development of new diagnosis for malignant progression in patients with NSCLC.

ACKNOWLEDGMENTS

We thank Dr. Yukito Ichinose (Kyushu Cancer Center, Fukuoka, Japan) and Kyogo Itoh (Kurume University, Fukuoka, Japan) for providing tumor cell lines.

REFERENCE


FIGURE 6. Correlation between N-myc downstream-regulated gene 1 (NDRG1)/Cap43 expression and microvascular density (MVD) in human lung cancer specimens. IHC analysis of microvessels in lung cancer samples using CD34 antibody. A, tumor areas with low and high vessel densities are evident in adenocarcinoma and squamous cell carcinoma. B, Kaplan-Meier analysis of overall survival in relation to MVD levels for adenocarcinoma (A left) and squamous cell carcinoma (B right). C, Correlation between NDRG1/Cap43 expression and MVD. In patients with adenocarcinoma and squamous cell carcinoma, the median value of MVD in NDRG1/Cap43-negative specimens was 2793 and 731, and in NDRG1/Cap43-positive specimens was 4370 and 1146, respectively. IHC, immunohistochemistry.


