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ORIGINAL ARTICLE

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

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Abstract: 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. 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 NGRG1/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. 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.

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N-myc downstream-regulated gene 1 (NDRG1)/Cap43 has been identified as a nickel- and calcium–inducible gene,[1,2] and is identical to both homocysteine-inducible gene reduced in tumor cells (RTP/rit42),[3] and differentiation-related gene-1(Drg-1).[4] 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 VHL).[1,3,5–9] 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.[5,10–12]

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.[13] 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.[14–16] In contrast, high NDRG1/Cap43 expression is related to poor prognosis and angiogenesis in cervical adenocarcinoma and gastric cancer.[17,18] 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.[19] Thus it seems that whether high NDRG1/Cap43 expression suppresses tumor growth and angiogenesis depends upon the type of human malignancy.[20]

Lung cancer continues to be the leading cause of cancer deaths worldwide.[21] Non–small-cell lung cancer (NSCLC) is the most common type of lung cancer, accounting for

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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.[22–24] 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.[25] 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 cells lines A549, PC9, 11_18, LK87, LC-1 (adenocarcinoma), QG56, LC-Sq-1, and RERF-LC-AI (squamous cell carcinoma) were maintained in RPMI1640 supplemented with 10% fetal bovine serum (FBS) and incubated in a humidified atmosphere of 5% CO_2 at 37°C. Anti-NDRG1/Cap43 antibodies were produced in our laboratory.[9,26] 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 AQ4 by Kyogo Itoh (Kurume University, Fukuoka, Japan). The rabbit polyclonal antibody against NDRG1/Cap43, which 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 Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), anti-c-Met was obtained from IBL Laboratories (Gunma, Japan), and anti-beta-actin was pur-AQ5 chased from Sigma Aldrich.

Small Interfering RNA Transfection and Immunoblotting

Small interfering RNA (siRNA) corresponding to a nucleotide sequence of NDRG1/Cap43 (5′-AAC GTG AAC CCT TGT GCG GAA-3′) was purchased from QIAGEN Inc. (Valencia, CA). A negative control siRNA was obtained AQ6 from Dharmacon. siRNA duplexes were transfected using LipofectAMINE RNAiMAX and Opti-MEM medium AQ7 (Invitrogen) in accordance with the manufacturer's recommendations. Seventy-two hours after siRNA transfection, the cells were lysed in cold protein extraction reagent (M-PER; Pierce) with both protease and phosphatase inhibitors. Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using NuPage 4% to 12% Bis-Tris gels (Invitrogen) in accordance with the manufacturer's instructions, and Western blot analyses were performed using a standard protocol. After transfer, the blots were incubated with blocking solution and probed with antibodies. The intensity of the luminescence was quantified using a CCD camera combined with an image analysis system (LAS-4000; Fuji Film, Japan).

NDRG1 Knockdown Vector Construction and Transfection

Cap43 complementary DNA was amplified by reverse transcription-polymerase chain reaction using the 5′and 3′primers5′-GATCCGCGTGAACCCTTGTGCGGAATTCA AGAGATTCCGCACAAGGGTTCACGTTTTTTGGAA-3′ and 5′-AGCTTTTCCAAAAAACGTGAACCCTTGTGCGG AATCTCTT

GAATTCCGCACAAGGGTTCACGCG-3′, respectively. The amplified Cap43 complementary DNA was then ligated into the pcDNA3_GFP_hU6siRNA 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/10^5 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 AQ8 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 $LW^2/2$. 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.

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Nude Mouse Xenograft Models and Determination of MVD

Cells were suspended in sterile phosphate-buffered saline at a concentration of 10^8 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.[13] 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 antihuman 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 (1×10^6 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.[28] 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 (Nikon, DXM1200). AQ9 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-positve 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

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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. 1*A*). 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. 1*B* 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. 1*B* 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. 2*A*). A549/Sic-9 showed decreased HER3 expression relative to A549/mock5, but no apparent changes in the expression of other growth factor receptors (Fig. 2*B*). 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. 2*B*). 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

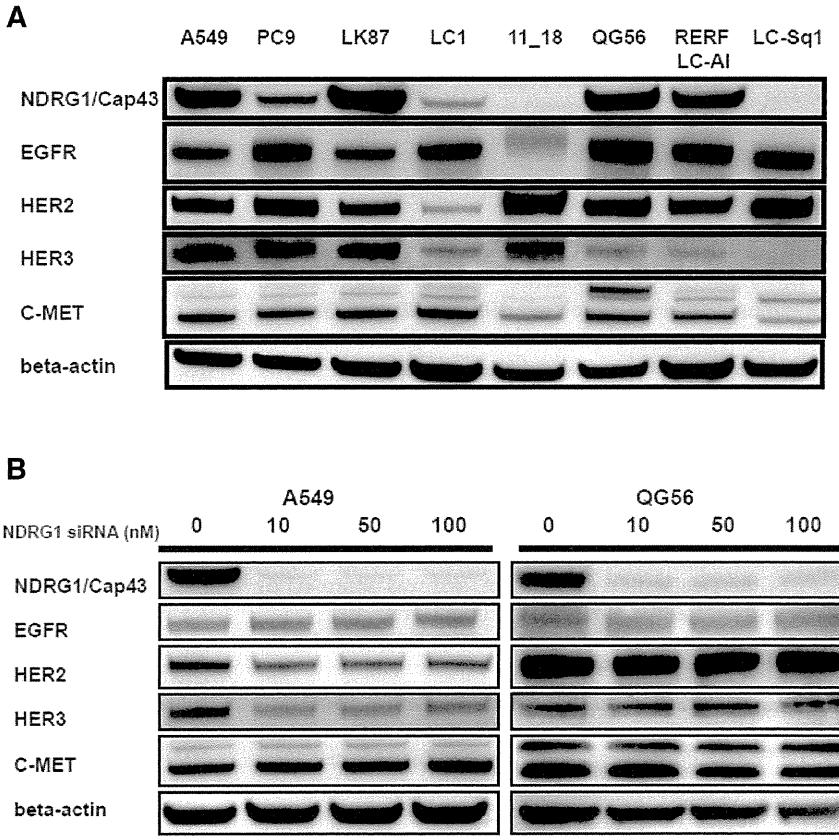

FIGURE.1. Lack of correlation between NDRG1/Cap43 expression level and expression of EGFR, HER2, HER3, and c-MET in human lung cancer. *A*, Expression of NDRG1/Cap43 in seven cell lines was determined by Western blot analysis in a human lung cancer cell line panel. *B*, A549 and QG56 cells were treated 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.

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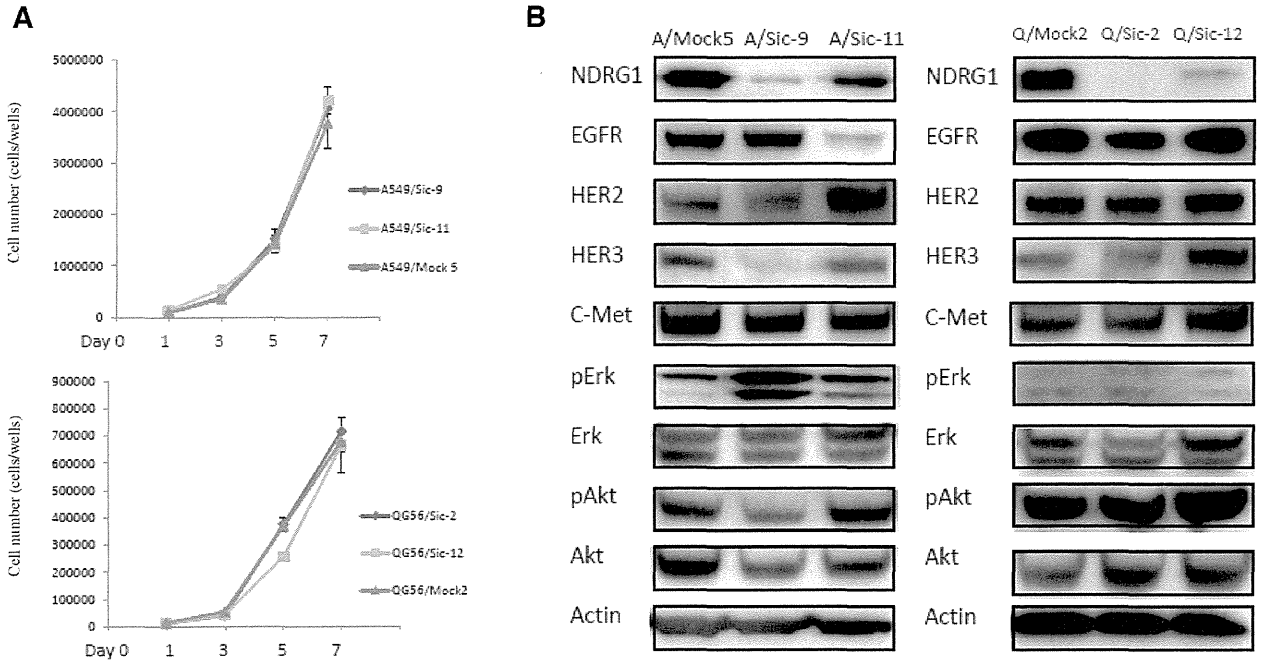

FIGURE 2. Lack of effect of NDRG1 knockdown on cell proliferation in vitro. *A*, Comparison of cell growth between NDRG1 siRNA transfectants and mock transfectants in A549 and QG56. Cell proliferation in RPMI containing 10% FBS was measured on days 1, 3, 5, and 7. Columns indicate mean values from three independent experiments; bars represent SD. *B*, Expression of NDRG1/Cap43 in NDRG1 siRNA transfectants or mock transfectants in two lung cancer cell lines determined by Western blot analysis. NDRG1, N-myc downstream-regulated gene 1; FBS, fetal bovine serum; siRNA, Small interfering RNA. AQ14

expression of other growth factors between QG56/Sic-2 and QG56/Sic-12 relative to those in QG56/Mock2 (Fig. 2*B*). 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, PAkt, ERK, and PERK between NDRG1/Cap43-knockdown 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. AQ10

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 cancer[18] 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. F3

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. 4*A*). 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. 4*B*). A549/Sic-9 tumors showed less development of neovessels than A549/Mock-5 tumors (Fig. 4*B*). 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. 4*C*). F4

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

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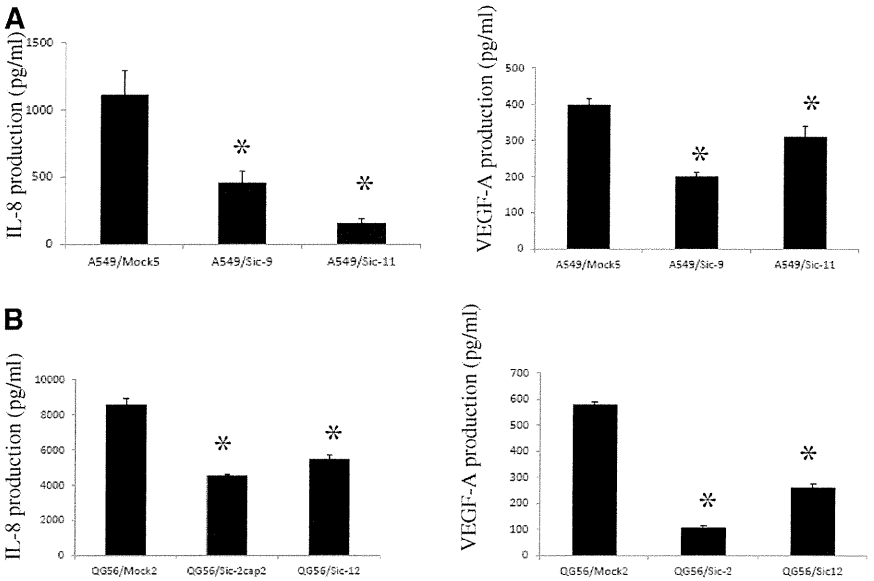

FIGURE 3. Effects of NDRG1/Cap43 knockdown on expression of VEGF-A and IL-8/CXCL8. *A* and *B* Comparison of 24-hour VEGF-A and IL-8 protein production between NDRG1-knockdown cell lines and their parental counterpart by ELISA assay in A549 (*A*) and QG56 (*B*). Columns represent mean values from triplicate experiments; bars represent SD. *$p < 0.05$: Comparison between NDRG1/Cap43 siRNA versus mock transfectants. NDRG1, N-myc downstream-regulated gene 1; VEGF-A, vascular endothelial growth factor-A; IL-8, interleukin-8; ELISA, enzyme-linked immunosorbent assay, siRNA, small interfering RNA.

and curled microvessels with tiny bleeding spots, in addition to the preexisting vessels (Fig. 4*D*), 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.4*E*).

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 5*A* 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 5*C*, 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 either adenocarcinoma (p=0.637; 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. 6*B*). Contrary to this, even after adjustment for MVD and age, patients with high nuclear expression of NDRG1/

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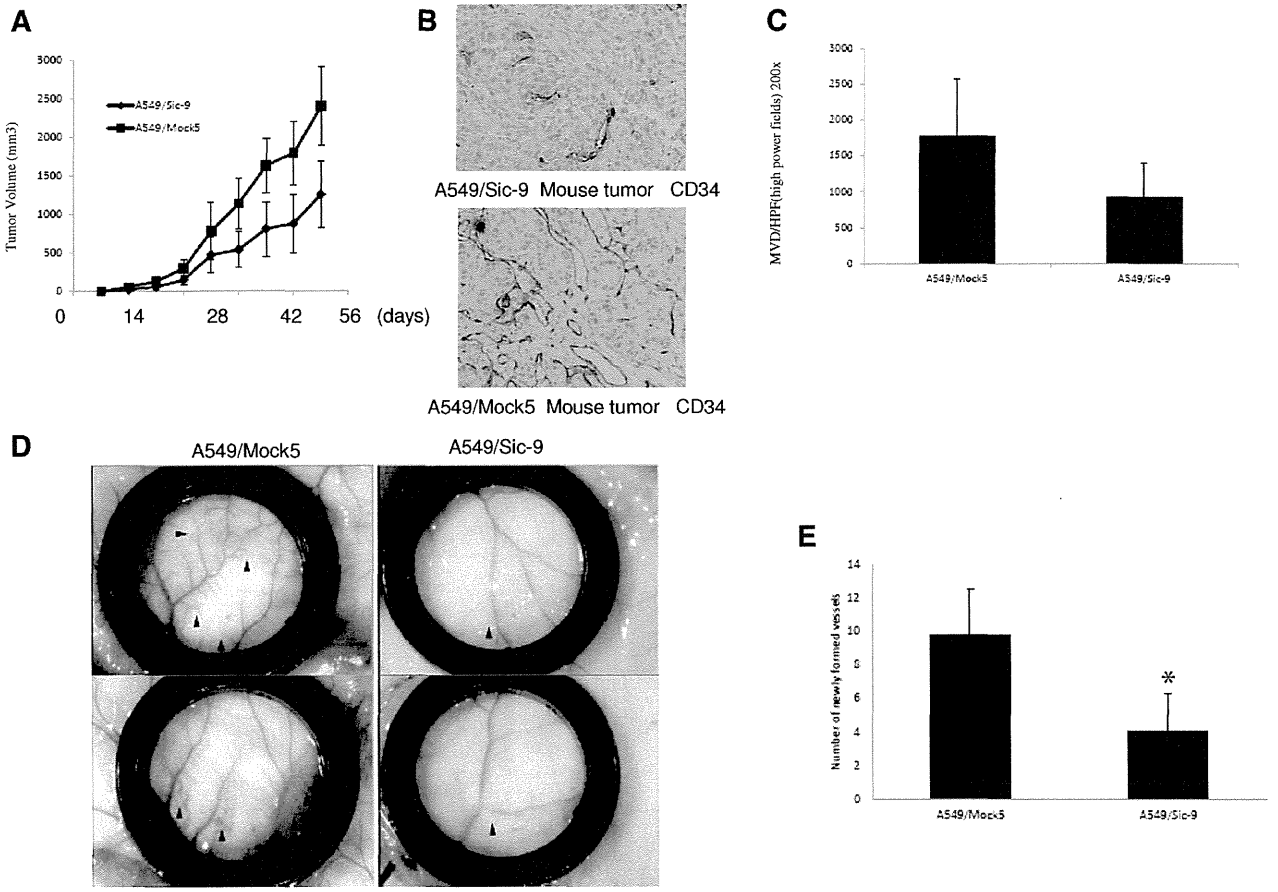

FIGURE 4. Comparison of tumor growth and angiogenesis between NDRG1/Cap43 siRNA transfectants and corresponding mock transfectants. *A*, mean tumor volumes with SD for groups of A549/sic-9 or A549/Mock5 inoculated with 1 × 10^7 cells. Tumor volumes were determined every week. *B*, Representative photographs of tumor sections stained with anti-CD34 (microvascular density) from A549/sic-9 and A549/Mock5. *C*, The mean microvessel densities for tumor sections from A549/sic-9 and A549/Mock5 were determined by counting the number of CD34-positive vessels in high-power fields of each section. Columns show mean values with SD from three independent experiments. *D*, Representative photographs of assay chambers containing NDRG1 siRNA transfectants and mock transfectants. *E*, The angiogenic responses were evaluated by counting the number of new blood vessels. Columns indicate mean values with SD from independents experiments. *$p<0.05$ significant difference between NDRG1 siRNA and mock transfectants. NDRG1, N-myc downstream-regulated gene 1; siRNA, small interfering RNA.

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 6*A*.

Figure 6*C* shows the distributions of MVD using boxplots 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. 6*C*). Expression of NDRG1/Cap43 in the cytoplasm was not associated with MVD in either adenocarcinoma or squamous cell carcinoma (data not shown).

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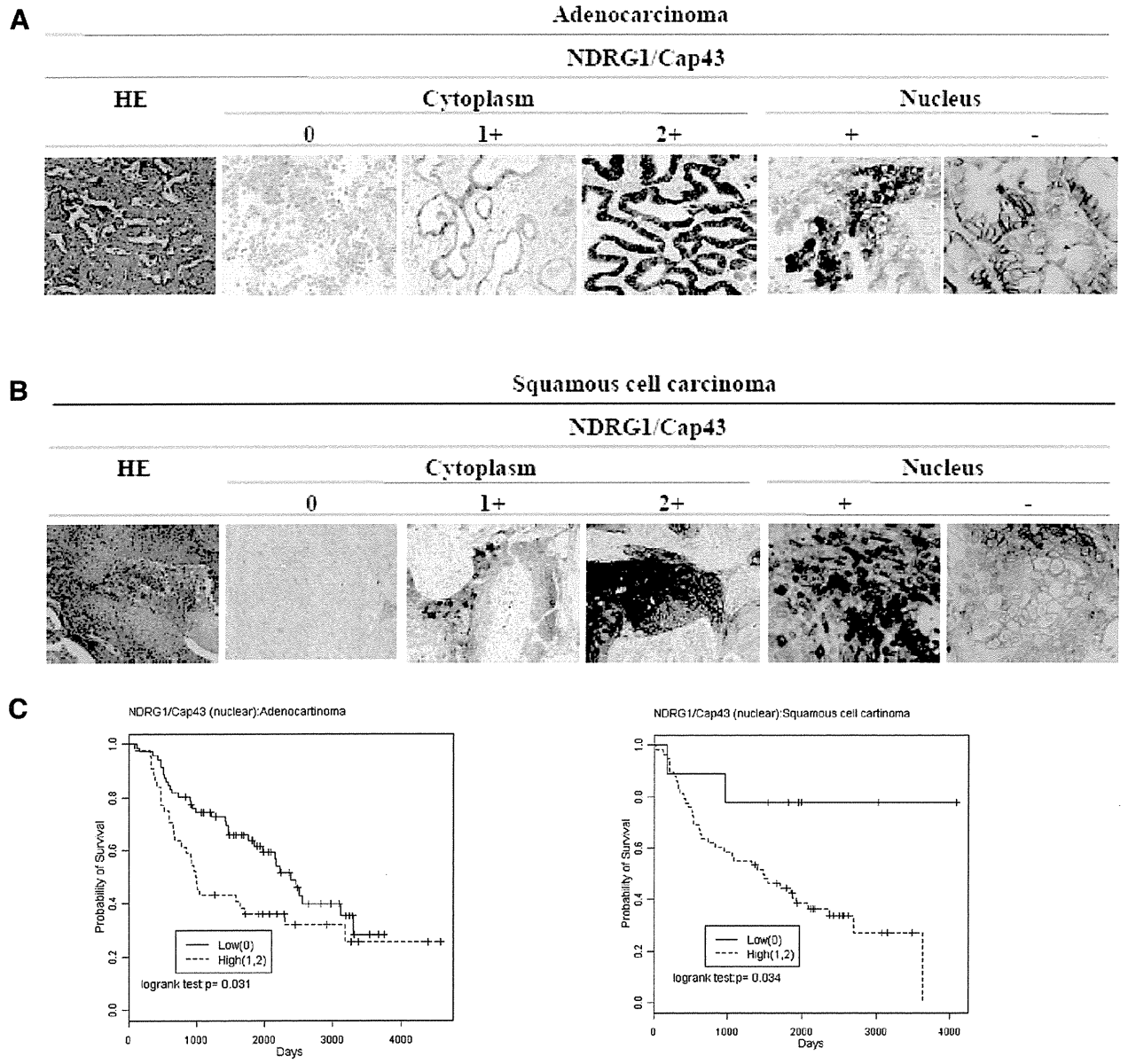

FIGURE 5. Expression of NDRG1/Cap43 in human lung cancer specimens analyzed by immunohistochemistry (IHC). *A and B,* Nuclear and cytoplasmic expression of NDRG1/Cap43 can be seen in the tumor cells of adenocarcinoma (*A*) and squamous cell carcinoma (*B*). *C,* Kaplan-Meier analysis of overall survival 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.

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. 5*A, 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.[13–16,29,30] By contrast, NDRG1 expression is a predictive marker of poor outcome in patients with liver, cervical, and gastric cancer.[17–19] Our present study indicated

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

				NDRG1/Cap43 (nuclear)			NDRG1/Cap43 (cyto)		
Histological type	**Characteristics**		**Number**	**Low**	**High**	***p* value**	**Low**	**High**	***p* value**
Adenocarcinoma N=115	Age	−64	56	30 (53.60%)	26 (46.40%)	0.0877	19 (33.90%)	37 (66.10%)	0.0388
		65−	59	41 (69.50%)	18 (30.50%)		32 (54.20%)	27 (45.80%)	
	Sex	F	60	37 (61.70%)	23 (38.30%)	1	23 (38.30%)	37 (61.70%)	0.193
		M	55	34 (61.80%)	21 (38.20%)		28 (50.90%)	27 (49.10%)	
	pStage	I	44	27 (61.40%)	17 (38.60%)	0.7427	19 (43.20%)	25 (56.80%)	0.9304
		II	19	10 (52.60%)	9 (47.40%)		8 (42.10%)	11 (57.90%)	
		III	36	23 (63.90%)	13 (36.10%)		17 (47.20%)	19 (52.80%)	
	Smoking status	Never	62	39 (62.90%)	23 (37.10%)	0.8483	23 (37.10%)	39 (62.90%)	0.1316
		Smoker	53	32 (60.40%)	21 (39.60%)		28 (52.80%)	25 (47.20%)	
Squamous cell carcinoma N=67	Age	−64	21	4 (19.00%)	17 (81.00%)	0.4458	10 (47.60%)	11 (52.40%)	0.7957
		65−	46	5 (10.90%)	41 (89.10%)		20 (43.50%)	26 (56.50%)	
	Sex	F	5	1 (20.00%)	4 (80.00%)	0.5255	2 (40.00%)	3 (60.00%)	1
		M	62	8 (12.90%)	54 (87.10%)		28 (45.20%)	34 (54.80%)	
	pStage	I	31	4 (12.90%)	27 (87.10%)	0.8087	13 (41.90%)	18 (58.10%)	0.949
		II	16	3 (18.80%)	13 (81.30%)		8 (50.00%)	8 (50.00%)	
		III	20	2 (10.00%)	18 (90.00%)		9 (45.00%)	11 (55.00%)	
	Smoking status	Never	4	1 (25.00%)	3 (75.00%)	0.4465	3 (75.00%)	1 (25.00%)	0.3179
		Smoker	63	8 (12.70%)	55 (87.30%)		27 (42.90%)	36 (57.10%)	

NDRG1, N-myc downstream-regulated gene 1.

TABLE 2. Multivariate Analysis of Overall Survival

	***p* Value**	**HR**	**95% CI**
NDRG1/Cap43 (nucleus) High/Low	0.0298	1.757	1.057–2.922
Tumor angiogenesis (MVD)	0.6916	0.929	0.647–1.335
Age	0.6026	0.993	0.969–1.018

HR, hazard ratio; CI, confidence interval; NDRG1, N-myc downstream-regulated gene 1; MVD, microvascular density.

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-kB pathway.[13,31] 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.[13,32,33] 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.[13,31] 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.[13,31] 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 MMP-9 in cultured pancreatic cancer cells.[13,31] AQ11
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.[13] However, our present study showed that NDRG1 exerted opposite effects on tumor angiogenesis

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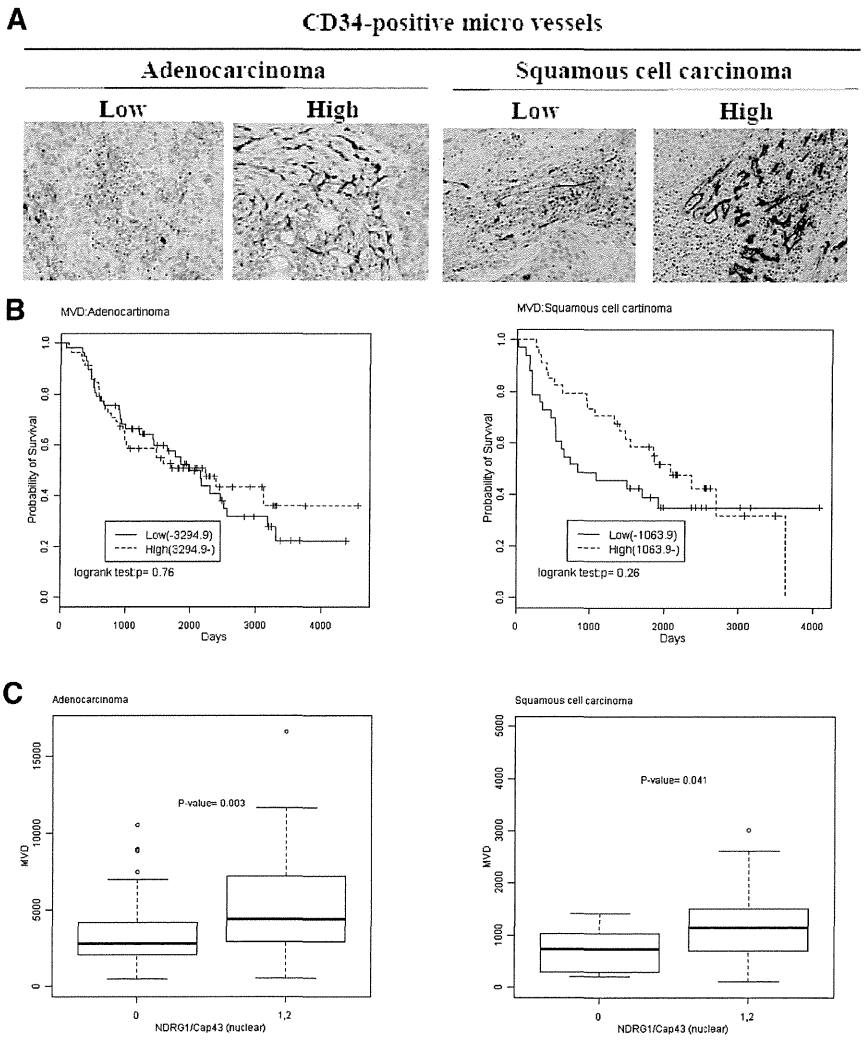

FIGURE 6. Correlation between N-myc downstream-regulated gene 1(NDRG1)/Cap43 expression and microvascular density(MVD) in human lung cancer specimens. IHC analysis of microvessels in lung cancer samples using CD34 antibody. *A,* tumor areas with low and high vessel densities are evident in adenocarcinoma 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.

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.[17] 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.[18] 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.

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Nocturia in Male Patients with Obstructive Sleep Apnea: Efficacy of Medication for Benign Prostatic Hypertrophy

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Abstract : ***Background and objectives:*** Nocturia, which is one of the symptoms in patients with benign prostatic hypertrophy (BPH), is also a well-known symptom in patients with obstructive sleep apnea (OSA). The prevalence of nocturia in OSA patients following nasal continuous positive airway pressure (nCPAP) treatment has not been investigated thoroughly, neither has the prevalence of BPH in OSA patients who need nCPAP treatment. Thus, the objective of this study was to investigate the overall prevalence of BPH that required treatment and the contribution of BPH or OSA to nocturia in OSA patients who needed nCPAP treatment.

Methods: Among 50 consecutive male patients with moderate to severe OSA over the age of 50 years who were treated by nCPAP treatment, 22 had been already examined by urologists. Therefore, 28 males with moderate to severe OSA were prospectively studied.

Results: NCPAP significantly decreased the number of nocturnal voids (2.4 ± 1.6 to 1.3 ± 1.0, p=0.0002) in 28 patients. In 18 of 28 patients requiring medication for BPH even after nCPAP treatment, the treatment further significantly decreased the number of nocturnal voids (1.5 ± 1.0 to 0.9 ± 0.8, p=0.026). Of the 22 patients seen by urologists before nCPAP, 12 had BPH requiring treatment. Thus, 30 of 50 patients (60%) had BPH requiring treatment.

Conclusions: BPH is prevalent in patients with moderate to severe OSA. Additional medication for BPH might be considered for the treatment of nocturia in them, when nCPAP is insufficient in treating their nocturia.

Key words : **Obstructive sleep apnea, Benign prostatic hypertrophy, Nocturia, Nasal continuous positive airway pressure**

Introduction

Obstructive sleep apnea (OSA) is characterized by repeated episodes of apnea during sleep. There is a growing body of evidence to support the belief that severe OSA is a risk factor for cardiovascular disease and death,[1)] not only in clinical cases[2),3)] but also in the general population.[4),5)] In addition to being one of the risk factors for cardiovascular disease, OSA induces several symptoms such as daytime sleepiness, insomnia, general fatigue, etc. Nocturia, which is one of the symptoms in

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patients with benign prostatic hypertrophy (BPH), is also a well-known symptom in patients with OSA.[6] It is believed that nocturia due to OSA may be relieved by nasal continuous positive airway pressure (nCPAP), the first-line treatment for OSA. However, the prevalence of nocturia in OSA patients following nCPAP treatment has not been investigated, thoroughly neither has the prevalence of BPH in OSA patients who need nCPAP treatment. It has been reported that nocturia produces insomnia, disturbs quality of life, and also affects mortality.[7] Therefore, it was important to investigate the overall prevalence of BPH which required treatment and the contribution of BPH or OSA to nocturia in moderate to severe OSA patients who needed nCPAP treatment.

A previous study indicated that OSA contributes substantially to causing nocturia in some individuals under the age of 50.[8] The effect on nocturia of adding treatment for BPH to nCPAP in patients over the age of 50, with moderate to severe OSA, was not clear. The current study hypothesized that both OSA and prostate diseases are associated with nocturia in those patients with OSA. To test the hypothesis, consecutive patients with OSA who were treated with nCPAP treatment were prospectively investigated.

Subjects And Methods

1 Subjects

Subjects were recruited from 55 consecutive men over the age of 50 with OSA who were admitted from August 2003 to July 2006 to the Department of Respiratory Medicine, Fukuoka University Hospital. These patients had an apnea-hypopnea index (AHI) of 20 or higher and received nCPAP. Excluded from the original 55 patients were as follow: 2 patients with brain natriuretic peptide (BNP)≥100 pg/ml before nCPAP and uncontrollable congestive heart failure; 2 patients who stopped nCPAP due to discomfort; 1 patient who used nCPAP less than 4 hours a day according to a memory card recorder. Thus, 50 patients were investigated. From these, 22 who had been previously seen by any urologists were excluded (Assessment 1, below). Therefore, the study population comprised 28 patients. This study was approved by the Ethics Committee of Fukuoka University. Informed consent was obtained from all participants.

2 Study protocol

Firstly, after carefully taking patients' past history and reviewing their clinical records, patients who had previously seen by urologists were excluded from further analysis (Assessment 1). Next, the eligible patients assessed the number of nocturnal voids at the first physical examination before introduction of nCPAP treatment (Assessment 2). Then, at 1 year after the start of nCPAP treatment, patients examined the number of nocturnal voids again and were seen by urologists regardless of whether they had nocturia or not, and also assessed their symptom using the International Prostate Symptom Score (IPSS)[9] (Assessment 3). When patients had to be seen by urologists for their nocturia at least two months after the start of nCPAP treatment due to intolerable voids, the number of nocturnal voids at this examination was used. Then, even if they were prescribed with BPH treatment or not by urologists in either case, the number of nocturnal voids using the IPSS was assessed one year after (Assessment 4).

3 OSA and PSG

All patients underwent PSG. The PSG system used was the Alice 4 (Respironics, Murrysville, PA, USA). Electroencephalogram (EEG; C3/A2, C4/A1, O1/A2), electrooculograms, chin electromyogram (EMG), tibialis anterior EMG, electrocardiogram (ECG), and chest and abdominal movements were recorded; at the same time, sensors were used to determine body position, air flow (thermistor), and oxygen saturation by pulse oximetry (SpO_2). The start of sleep and final wake-up times were determined by PSG. Waking EEG and sleep stages were determined by the criteria of Rechtschaffen & Kales.[10] Total sleep time was determined based on the EEG. Apnea was defined as a complete cessation of airflow at the nose and mouth that lasted for ≥10s. Hypopnea was defined as a ≥50% reduction in oronasal airflow for ≥10s, associated with a ≥3% fall in arterial oxygen saturation or an arousal.[11] AHI was the number of episodes of obstructive apnea, mixed apnea, and hypopnea per hour of sleep. The frequency of arousal due to apnea or hypopnea served as the arousal index. The lowest SpO_2 and the percentage of total sleep time of $SpO_2 < 90\%$ (%TST of $SpO_2 < 90\%$) were also calculated. Patients with an AHI greater than 20 underwent nCPAP treatment under the health insurance system in the Japanese government. Severity of OSA was defined by AHI as follows: no OSA (AHI<5/h), mild OSA (AHI=5-14.9/h), moderate OSA (AHI=15-29.9/h) and severe OSA (AHI≥30/h). In addition, nCPAP (REM Star: Respironics, Murrysville, PA, USA) titration was carried

out during the PSG.

4 Severity of BPH

The severity of BPH was diagnosed according to the guidelines of the Japanese Urological Association.[12),13)] The IPSS is an 8-question (7 symptom questions + 1 quality of life question) written screening tool for the symptoms of BPH.[9)] The number of nocturnal voids is included as one of their questions ("Over the past month, how many times did you typically get up to urinate from the time you went to bed at night until the time you got up in the morning?"). The severity of each factor was classified as mild, moderate, or severe by areas of assessment, i.e. symptoms, urinary function (maximum urinary flow rate and residual urine), and morphology (prostate volume).[12),13),14)] In addition, overall severity (mild, moderate and severe) was determined depending on the number of items to determine that severity.

5 Assessment of sleepiness

The modified Japanese version of the Epworth Sleepiness Scale (ESS)[15)] was used to assess subjective sleepiness.

6 Statistical analysis

Data are presented as mean ± standard deviation. The effects of nCPAP titration on various sleep parameters were determined with a paired-t test. The statistical significance of differences in nocturnal voiding between Assessment 2 and Assessment 3, and between Assessment 3 and Assessment 4 was determined with Wilcoxon signed-rank test. $P<0.05$ was considered significant.

Results

1 Clinical characteristics

Twenty-eight patients completed the protocol, and their characteristics are shown in Table 1. Their age was 65.5 ± 8.9 years old and their body mass index (BMI) was 25.5 ± 3.3kg/m^2. Six patients were current smokers, 19 were ex-smokers and the remaining 3 patients were never smokers. Eighteen (64%) had hypertension (systolic blood pressure (BP) ≥140mmHg and/or diastolic BP ≥90mmHg) and/or were being prescribed anti-hypertension drugs (no α blockers). Sixteen (57%) had total cholesterol concentration ≥220mg/dl and/or triglycerides ≥150mg/dl and/or high-density lipoprotein cholesterol <40mg/dl and/or were being prescribed a statin. Eight (29%) had type 2 diabetes, but none was receiving treatment for this condition, there were no complications, and HbA1c was controlled to under 6.5% in addition to no glucose in the fasting urine.

On the ESS, they had a score of 10.9 ± 5.6, indicating that mild daytime sleepiness was noted (Table 1). They had an AHI of 40.1 ± 14.9; 7 patients had moderate OSA and 21 had severe OSA. As a result of nCPAP titration, AHI improved significantly to 4.8 ± 5.0 ($p<0.0001$). Significant improvement was also noted in the arousal

Table 1. Characteristics of the 28 subjects

	Before nCPAP	During nCPAP titration	p value
Age, y	65.5 ± 8.9		
BMI, kg/m^2	25.5 ± 3.3		
ESS	10.9 ± 5.6		
AHI	40.1 ± 14.9	4.8 ± 5.0	<0.0001
Arousal index	37.4 ± 13.9	16.7 ± 8.6	<0.0001
REM stage, %	16.2 ± 7.7	17.6 ± 3.7	0.52
stage Ⅰ, %	42.0 ± 18.5	30.1 ± 17.5	0.054
stage Ⅱ, %	40.8 ± 15.9	49.8 ± 16.7	0.11
stage Ⅲ, %	0.7 ± 1.4	2.1 ± 3.1	0.025
stage Ⅳ, %	0.0 ± 0.0	0.0 ± 0.1	0.33
Lowest SpO_2, %	79.7 ± 7.1	90.2 ± 4.4	0.00019
%TST of SpO_2 < 90%, %	11.4 ± 15.1	2.3 ± 5.7	0.023
Snore index, %	23.9 ± 21.2	3.3 ± 8.4	0.00056

BMI: body mass index, ESS: Epworth Sleepiness Scale, AHI: apnea hypopnea index,
REM: rapid eye movement, SpO_2: oxygen saturation by pulse oximetry, TST: total sleep time

index, stage III sleep, lowest SpO_2, %TST of SpO_2 < 90% and snore index.

2 Severity of BPH and medication

Moderate to severe BPH was noted in 21 of 28 patients (75%) (mild in 7, moderate in 18, and severe in 3) at Assessment 3. The average prostate volume was 29.7 ± 16.2 ml. Eighteen patients received medication for BPH and 10 did not. Their medication was as follows: α1-blocker alone, 12 patients; phytotherapeutic agent alone, 3 patients; and their combination, 3 patients.

3 Effects of nCPAP on the changes in the number of nocturnal voids

For the 28 patients studied, the number of nocturnal voids decreased significantly with nCPAP from 2.4 ± 1.6 (Assessment 2) to 1.3 ± 1.0 (Assessment 3) (p=0.0002) (Figure 1.). The average duration of nCPAP treatment before visiting urologists was 10 months. Eighteen of the 28 patients required medication from urologists (Assessment 3). The other 10 patients did not take medications for BPH following the examinations of the urologists. Before the medication, the number of nocturnal voids in 18 patients decreased significantly as a result of nCPAP treatment, from 2.6 ± 1.7 (Assessment 2) to 1.5 ± 1.0 (Assessment 3) (p=0.0029). For the 10 patients who were not treated for BPH after they were seen by urologists, the number of nocturnal voids also decreased significantly after nCPAP treatment, from 1.9 ± 1.3 (Assessment 2) to 0.8 ± 0.8 (Assessment 3) (p=0.031) (Figure 1.).

4 Effects of medication for BPH

Following 1 year of the medications for BPH, the number of nocturnal voids in 18 patients further decreased from 1.5 ± 1.0 (Assessment 3) to 0.9 ± 0.8 (Assessment 4) (p=0.026) (Figure 1.). However, for the 10 patients who were not treated for BPH after they were seen by urologists, the number of nocturnal voids did not change significantly (0.8 ± 0.8 (Assessment 3) to 0.5 ± 0.8 (Assessment 4), p=0.083) following the next 1year of nCPAP treatment (Figure 1.).

5 Correlation between the number of nocturnal voids and physiological findings

The number of nocturnal voids prior to medication (Assessment 2) was not significantly correlated with age, PSG findings (AHI, arousal index, lowest SpO_2, %TST of SpO_2 < 90% and snore index) and prostate volume.

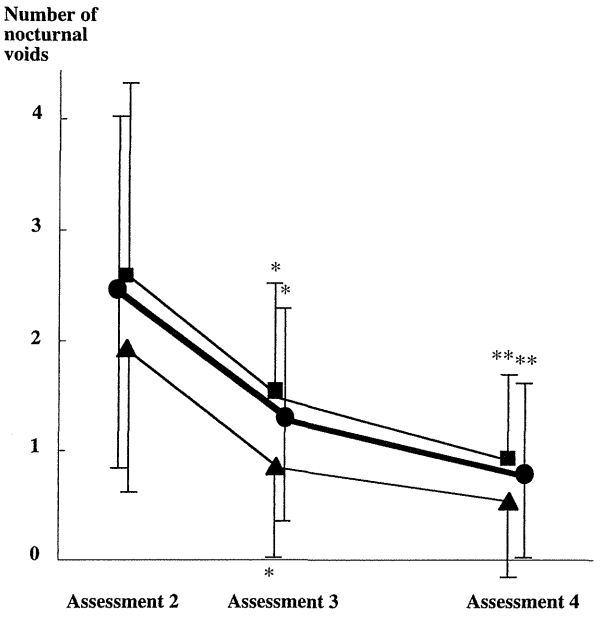

Figure 1. Changes in the number of nocturnal voids before nCPAP (Assessment 2), after nCPAP (approximately 10 months) (Assessment 3), and after checked by the urologists (approximately 12 months) (Assessment 4) in all 28 patients (●), 18 patients with BPH requiring its treatment (■), and 10 patients without BPH medication (▲). *p<0.05, versus Assessment 2; **p<0.05, versus Assessment 3.

6 Twenty-two patients who had already seen by urologists

Sixteen of the 22 patients who were excluded at Assessment 1 were diagnosed as BPH. Twelve of these 16 patients with BPH required treatment; 3 with surgery, 9 with medication, 2 with prostate cancer, 2 with ureteral calculi, 1 with prostatitis, and 1 with neurogenic bladder.

Of the 28 patients that completed the protocol, 18 had BPH requiring medication (Assessment 3). Thus, after all, among 50 patients who had entered the study, 30 patients (60%) had BPH requiring treatment.

Discussion

The current study examined male patients with moderate to severe OSA. We found out that (1) nocturia was frequently noted and the number of nocturnal voids decreased after nCPAP treatment within one year, (2) nocturia did not fully improve despite nCPAP treatment for approximately 10 months, and more than half of the

patients required additional medication for BPH, and (3) nocturia was managed better by nCPAP treatment and medication for BPH in these patients.

After middle age, nocturia is noted in many patients;[16] it diminishes their quality of life and is related to a reduced life expectancy.[7] Many patients with OSA have nocturia. Moriyama et al.[8] interviewed 73 men under the age of 50 with OSA about their nocturia, and noted that 41.1% had 2 or more voids after the start of asleep. This indicates that OSA may be a major cause of nocturia in men under the age of 50. Hajduk et al.[17] interviewed 138 patients with OSA and reported a frequency of nocturia of 47.8%; they also reported finding that AHI was positively correlated with nocturia, independent of BMI. Additionally, nocturia is reported to improve after nCPAP treatment for one night.[18),19] However, none of the previous studies has shown the effects of long-term nCPAP treatment on the number of nocturnal voids. In the present study, after 10-month nCPAP treatment, the number of nocturnal voids significantly decreased from 2.4 ± 1.6 (Assessment 2) to 1.3 ± 1.0 (Assessment 3). Two mechanisms have been proposed for nocturia in patients with OSA[20]: (1) diuresis increases as a result of an increase in atrial natriuretic peptide during sleep[21]; and (2) rapid changes in abdominal pressure during an episode of apnea directly affect bladder function, although atrial natriuretic peptide during sleep was not measured in this study.

We found that nCPAP did not eliminate nocturnal voids completely in some patients with OSA. The results in this study showed that nocturia in OSA males over 50 might be the result of BPH as well as factors related to OSA. In men, the frequency of BPH increases after middle age and lower urinary tract symptoms such as dysuria and frequent urination become manifest.[22] No previous reports have examined the degrees of contribution of OSA and BPH to nocturia. Then, current study prospectively studied the effects of nCPAP and medication for BPH on nocturia in men with OSA.

When patients were seen by urologists after long-term nCPAP treatment, medication for BPH was added for 18 of 28 patients (64%) based on Japanese guidelines for BPH. The core of the treatment is to reduce urethral obstruction due to an enlarged adenoma (mechanical obstruction) or ease constriction of the urethra (dynamic or functional obstruction) by α1-receptors in the sympathetic nervous system. In this study, additional medications for BPH significantly decreased the number of nocturnal voids from 1.5 ± 1.0 (Assessment 3) to 0.9 ± 0.8 times (Assessment 4). Following the BPH medications, the number of nocturnal voids in patients with nCPAP plus medication reached nearly the same level of the patients with nCPAP but without BPH medication at Assessment 3 (0.8 ± 0.8 times). Thus, nocturia improved after administration of BPH medication (Figure 1.).

A high percentage of the current subjects, 75%, also had moderate to severe BPH. Masumori et al.[23] analyzed the results of routine examinations of the general population and found that the average volume of the prostate in a typical Japanese man in his 60s and 70s is about 21 or 22 ml. In Japan, a prostate volume of 20 ml or more is considered abnormal.[23),24] In the current study, the volume of the prostate was rather large at 29.7 ± 16.2 ml. Indeed, 30 out of 50 OSA patients (60%) receiving nCPAP treatment also had BPH requiring treatment. Thus, factors related to OSA and BPH were involved in causing nocturia in OSA males over 50. However, the severity of OSA did not directly correlate with prostate volume and the number of nocturnal voids. In patients with OSA, sympathetic activation is elevated in association with faster heart rates, decreased heart rate variability and increased blood pressure variability.[25] This might indirectly worsen BPH and cause increased prostate volume through obstructing the bladder outlet or urethra by contraction of smooth muscle.

Nocturia is also known to be caused by conditions like diabetes and urinary tract infections.[20] In the current study, 8 patients also had type 2 diabetes, but treatment was not administered to any of the patients, there were no complications, and HbA1c was controlled to under 6.5%. In addition, all patients underwent urinalysis, and urinary tract infections like cystitis were ruled out. Thus, we assumed that nocturia due to type 2 diabetes and cystitis was excluded in the current subjects.

This study has several limitations. First, the sample size was small. A further prospective large-scale study is needed. Second, the observation period prior to being seen by urologists was not uniform from 2 to 12 months. However, it would be unethical not to consult urologists for a long time in some patients suffering from severe voids.

In conclusion, BPH is prevalent in patients with moderate to severe OSA. We found that nCPAP alone may be insufficient to treat nocturia for some of patients with OSA. Combining nCPAP treatment and medication for BPH might be considered for the treatment of nocturia

in patients with OSA over the age of 50, when nCPAP is insufficient in treating their nocturia.

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Pro-apoptotic effects of imatinib on PDGF-stimulated pulmonary artery smooth muscle cells from patients with idiopathic pulmonary arterial hypertension ☆

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ABSTRACT

Background: Remodeling of the pulmonary artery by an inappropriate increase of pulmonary artery smooth muscle cells (PASMCs) is problematic in the treatment of idiopathic pulmonary arterial hypertension (IPAH). Effective treatment that achieves reverse remodeling is required. The aim of this study was to assess the pro-apoptotic effects of imatinib, a platelet-derived growth factor (PDGF)-receptor tyrosine kinase inhibitor, on PASMCs obtained from patients with IPAH.

Methods: PASMCs were obtained from 8 patients with IPAH undergoing lung transplantation. Cellular proliferation was assessed by ^{3}H-thymidine incorporation. Pro-apoptotic effects of imatinib were examined using TUNEL and caspase-3,7 assays and using transmission electron microscopy.

Results: Treatment with imatinib (0.1 to 10 μg/mL) significantly inhibited PDGF-BB (10 ng/mL)-induced proliferation of PASMCs from IPAH patients. Imatinib (1 μg/mL) did not induce apoptosis in quiescent IPAH-PASMCs, but it had a pro-apoptotic effect on IPAH-PASMCs stimulated with PDGF-BB. Imatinib did not induce apoptosis in normal control PASMCs with or without PDGF-BB stimulation. PDGF-BB induced phosphorylation of Akt at 15 min, and Akt phosphorylation was inhibited by imatinib in IPAH-PASMCs. Akt-I-1/2 (1 μmol/L), an Akt inhibitor, in the presence of PDGF-BB significantly increased apoptotic cells compared with the control condition. Thus, Akt-I-1/2 could mimic the effects of imatinib on PASMCs.

Conclusion: Imatinib has anti-proliferative and pro-apoptotic effects on IPAH-PASMCs stimulated with PDGF. The inhibitory effect of imatinib on Akt phosphorylation induced by PDGF plays an important role in the pro-apoptotic effect.

1. Introduction

Idiopathic pulmonary arterial hypertension (IPAH) is a progressive disease characterized by progressive elevation of pulmonary vascular resistance and pulmonary artery pressure. Increased pulmonary vascular resistance is induced by pulmonary vasoconstriction, vascular remodeling by intimal and medial hypertrophy, and thrombosis [1,2]. Pulmonary vascular medial hypertrophy is caused by an inappropriate increase in pulmonary artery smooth muscle cells (PASMCs). Treatment with several vasodilators such as calcium channel blockers, prostaglandin I_2 and endothelin receptor antagonists was found to improve survival of patients with IPAH, but 5-year survival remains at 50% [3,4]. Effective treatment that achieves reverse remodeling is needed. This will require anti-proliferative and pro-apoptotic agents for PASMCs.

We have reported that platelet-derived growth factor (PDGF)-BB stimulation causes a higher growth rate of cultured PASMCs from patients with IPAH than that of control cells [5–7]. Recently, the use of a PDGF-receptor inhibitor such as imatinib (STI571) is starting to garner attention as a targeted therapy for pulmonary hypertension (PH) [8–11]. Imatinib is a drug used to treat certain types of cancer such as chronic myelogenous leukemia and gastrointestinal stromal tumors. In laboratory settings, imatinib is used as an experimental agent to suppress PDGF by inhibiting PDGF receptor ß (PDGF-Rß). It is an agent that acts by specifically inhibiting a certain enzyme, tyrosine kinase, that

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