N-myc Downstream Regulated Gene 1/Cap43 Suppresses Tumor Growth and Angiogenesis of Pancreatic Cancer through Attenuation of Inhibitor of κB Kinase β Expression

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Abstract

N-myc downstream regulated gene 1 (NDRG1)/Cap43 expression is a predictive marker of good prognosis in patients with pancreatic cancer as we reported previously. In this study, NDRG1/Cap43 decreased the expression of various chemotactic cytokines, including CXC chemokines for inflammatory cells, and the recruitment of macrophages and neutrophils with suppression of both angiogenesis and growth in mouse xenograft models. We further found that NDRG1/Cap43 induced nuclear factor-κB (NF-κB) signaling attenuation through marked decreases in inhibitor of κB kinase (IKK) β expression and IκBα phosphorylation. Decreased IKKβ expression in cells overexpressing NDRG1/Cap43 resulted in reduction of both nuclear translocation of p65 and p50 and their binding to the NF-κB motif. The introduction of an exogenous IKKβ gene restored NDRG1/Cap43-suppressed expression of melanoma growth-stimulating activity α/CXCL1, epithelial-derived neutrophil activating protein-78/CXCL5, interleukin-8/CXCL8 and vascular endothelial growth factor-A, accompanied by increased phosphorylation of IκBα in NDRG1/Cap43-expressing cells. In patients with pancreatic cancer, NDRG1/Cap43 expression levels were also inversely correlated with the number of infiltrating macrophages in the tumor stroma. This study suggests a novel mechanism by which NDRG1/Cap43 modulates tumor angiogenesis/growth and infiltration of macrophages/neutrophils through attenuation of NF-κB signaling. [Cancer Res 2009;69(12):4983–91]

Introduction

N-myc downstream regulated gene 1 (NDRG1)/Cap43 is one of the metastasis suppressor genes (1), and expression of NDRG1/Cap43 is regulated by oncogenes (N-myc and C-myc) and tumor suppressor genes (p53, VHL, and PTEN; ref. 2). Expression of NDRG1/Cap43 protein is often elevated in many types of human tumors. In human cancer, expression of NDRG1/Cap43 depends on tumor type and differentiation status (2). Consistent with this idea, NDRG1/Cap43 expression in cancer cells is a predictive marker of good prognosis in patients with neuroblastoma or cancers of the prostate, breast, esophagus, colon, and pancreas (3–10), whereas its expression is a predictive marker of poor prognosis in patients with liver and cervical cancer (11, 12).

We previously identified NDRG1/Cap43 as one of the nine genes that are highly expressed in cancerous regions of human renal cell carcinoma (13), and its expression is closely associated with the VHL oncosuppressor gene (14). We further showed that expression of NDRG1/Cap43 is associated with a marked decrease of tumor angiogenesis in mice bearing human pancreatic cancer xenografts and that NDRG1/Cap43 markedly suppresses the expression of matrix metalloproteinase-9, vascular endothelial growth factor (VEGF), and interleukin (IL)-8/CXCL8. Moreover, expression of NDRG1/Cap43 has been associated with decreased microvessel density (MVD) and differentiation or depth of invasion in cancer cells with pancreatic cancer (5).

In the present study, we further examined how NDRG1/Cap43 modulates tumor growth and angiogenesis in pancreatic cancer. Because microarray analysis in this study and our previous studies showed that expression of some angiogenesis- and inflammation-related factors were markedly down-regulated by NDRG1/Cap43, we hypothesized that inflammation could be somehow associated with the NDRG1/Cap43-induced suppression of tumor growth and angiogenesis. Our results indicated that down-regulation of CXC chemokines and VEGF expression by NDRG1/Cap43 was actively involved in its suppression of angiogenesis and growth in pancreatic cancer as well as infiltration of macrophages and neutrophils, and we discuss whether attenuation of nuclear factor-κB (NF-κB) signaling plays a key role in this process.

Materials and Methods

Materials and cell lines. MIApaca-2 transfectants (Mock#2, Cap#11 and Cap#14) were maintained in DMEM supplemented with 10% fetal bovine serum and G418. The anti-NDRG1/Cap43 antibody was generated as described previously (5). Other antibodies were purchased as follows: anti-β-actin antibody (Abcam); anti-NIK, anti-TAK1/2, anti-Inhibitor of κB kinase (IKK) α, anti-IKKγ, anti-IKKβ, anti-p-IκBα, anti-p-65, anti-p50, anti-RelB, anti-p52, and anti-ubiquitin antibodies (Cell Signaling Technology); anti-p65 and anti-p50 antibodies for supershift analysis by electromobility shift assay (EMSA; Santa Cruz Biotechnology); anti-Flag M2 antibody (Sigma); and anti-CD68 and anti-neutrophil elastase antibodies (DAKO). Human tumor necrosis factor-α (TNF-α) and GM-132 were purchased from R&D Systems and Calbiochem.
Plasmid constructs. To obtain full-length cDNA of human IKKβ, PCR was carried out on a SuperScript cDNA library (Invitrogen) using the following primer pairs: 5′-ATAGGCTGCTGATCACTCACTG-3′ and 5′-TCATGAGGGCTGTCCTCCACCA-3′ (IKKβ). The PCR product was ligated into the pGEM-T Easy vector (Promega), and Flag-IKKβ was ligated into the p3xFLAG-CMV10 vector (Sigma).

Oligonucleotide microarray analysis. Duplicate samples were prepared for microarray hybridization. Total RNA (2 μg) was reverse transcribed using a GeneChip 3′-Amplification Regent’s One Cycle cDNA Synthesis kit (Affymetrix) and labeled with Cy5 or Cy3. The labeled cRNA was applied to the oligonucleotide microarray (Human Genome U133 Plus 2.0 Array; Affymetrix), the microarray was scanned on a GeneChip Scanner 3000, and the image was analyzed using GeneChip Operating Software version 1 as described previously (15).

Determination of melanoma growth-stimulating activity α/CXCL1, epithelial-derived neutrophil activating protein-78/CXCL5, IL-8/CXCL8, and VEGF-A levels by ELISA. The concentrations of IL-8/CXCL8, VEGF-A, melanoma growth-stimulating activity α (Gro)/CXCL1, and epithelial-derived neutrophil activating protein-78 (ENA-78)/CXCL5 in the homogenized supernatant of mouse xenograft tumors and conditioned medium were measured using commercially available ELISA kits (R&D Systems) in accordance with the manufacturer’s instructions.

EMSA. EMSA was done as follows. Nuclear extract (6 μg) was incubated for 15 min at room temperature with a 1 × 10^6 counts/min 32P-labeled oligonucleotide probe in binding buffer [10 mmol/L HEPES-NaOH (pH 7.9), 1 mmol/L EDTA, 50 mmol/L NaCl, 10% glycerol, 0.1 mg/mL bovine serum albumin, 0.05% NP-40, 0.005 mg/mL DTT, 0.05 mg/mL poly[d(eodeoxyinosin- deoxyctydyllic acid)] as described previously (16). The reaction mixtures were separated on a nondenaturing 4% polyacrylamide gel, and radioactivity was detected with a FLA 5000 image analyzer (Fuji Film).

Immunoprecipitations and Western blotting. The cells treated with or without MG-132 (10 μmol/L) under 2% serum condition for 8 h were lysed in lysis buffer [50 mmol/L Tris-HCL (pH 8.0), 250 mmol/L NaCl, 0.3% NP-40, 1 mmol/L EDTA, 10% glycerol, 0.1 mmol/L Na3VO4] supplemented with a mixture of protease inhibitors. Lysates were incubated with anti-ubiquitin antibody for 2 h at 4°C with protein A/G agarose for additional 1 h. After all immunoprecipitates were washed three times with lysis buffer, Western blotting was done with anti-IKKβ as described previously (17). The intensity of the luminescence was quantified using a FLA 5000 image analyzer (Fuji Film).

Animals. All animal experiments were approved by the Ethics of Animal Experiments Committee at Kyushu University Graduate School of Medical Sciences. Male athymic nu/nu mice were purchased from Charles River Laboratories and housed in microisolator cages maintained under a 12-h light/dark cycle. Water and food were supplied ad libitum. 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.

Immunohistochemical analysis. MIApaca-2 transfectants were injected subcutaneously into mice (1.0 × 10^7 cells/0.1 mL/mouse). At day 49 after transplantation of MIApaca-2 transfectants, the tumors were fixed and immunohistochemical analysis was done as described previously (5, 18). All human tissue samples were fixed and embedded in paraffin, and immunohistochemical analysis was done as described previously (5, 18). In all tissue samples, the mean value of the number of infiltrating macrophages and neutrophils and the MVD were calculated from four or five hotspots. All counts were done by three independent observers.

Statistical analysis. Data are expressed as mean ± SD. All calculations (Welch’s t test, Student’s t test, and Wilcoxon/Kruskal-Wallis test) were done using JMP version 5.0 (SAS Institute).

Patients and specimens. Surgically respected specimens from 37 patients with pancreatic ductal adenocarcinoma were studied. All patients underwent surgical resection between 1991 and 1998 at the Department of Surgery, Kurume University Hospital. Informed consent was obtained from all patients, and the study protocol was approved by the Ethics Committee of Kurume University.

Results

NDRG1/Cap43 down-regulates the expression of angiogenesis- and inflammation-related genes. To understand how NDRG1/Cap43 modulates tumor angiogenesis and growth in pancreatic cancer cells, we compared the expression profiles of NDRG1/Cap43 transfectant (Cap#11) and the parental low-expression counterpart (Mock#2) of MIApaca-2 cells using a high-density oligonucleotide microarray (Supplementary Table S1).

We selected eight genes predicted to be associated with adhesion, growth, and chemotaxis (Supplementary Table S2). Because our previous study showed that NDRG1/Cap43 overexpression in pancreatic cancer cells reduced the expression of angiogenesis-related factors such as VEGF-A and IL-8/CXCL8 (5), we also selected these genes, the expression of which showed a decrease of ~0.7 (Supplementary Table S2).

We confirmed the expression of NDRG1/Cap43 in two NDRG1/Cap43 transfectants (Cap#11 and Cap#14) and their mock transfectants (Mock#2) of MIApaca-2 cells (Fig. 1A). We compared the expression of these genes in high- and low-NDRG1/Cap43 expressing MIApaca-2 cells by quantitative real-time PCR. From the array results, we selected NCA1, which was up-regulated by NDRG1/Cap43, as a control. The mRNA expression levels of Gro/CXCL1, Ena/CXCL5, IL-8/CXCL8, and VEGF-A were significantly decreased in two NDRG1/Cap43 transfectants (Cap#11 and Cap#14) in comparison with Mock#2 cells (Supplementary Fig. S1).

We used ELISA assays to compare protein levels of chemokines among pancreatic cancer cells showing low and high expression of NDRG1/Cap43 (Fig. 1B). We observed that Cap#11 and Cap#14 cells showed a marked decrease in the production of Gro/CXCL1 and Ena/CXCL5 as well as IL-8/CXCL8.

NDRG1/Cap43 suppresses the NF-κB signaling pathway in pancreatic cancer cells. Representative angiogenic factors such as IL-8/CXCL8 and VEGF-A are regulated by NF-κB (19). We investigated whether NDRG1/Cap43 expression interfered with the NF-κB signaling pathway in pancreatic cancer cells. The phosphorylation of IκBα was activated in Mock#2 cells cultured in the presence of 2% serum compared with Cap#11 and Cap#14 cells (Fig. 1C, left). By contrast, in the absence of serum, there appeared to be weak activation of IκBα in Mock#2 cells. However, NDRG1/Cap43 expression level was not affected with or without serum in NDRG1/Cap43 transfectants (Fig. 1C, left). Next, we determined the expression levels of proteins related to the NF-κB signaling pathway to examine which molecules are responsible for the difference in the phosphorylation level of IκBα between NDRG1/Cap43 and mock transfectants. Phosphorylation of IκBα is regulated by the IKK complex, which consists of two catalytic subunits, IKKα and IKKβ, and a regulatory component, IKKy/NEO. The expression of IKKβ was markedly reduced in Cap#11 and Cap#14 cells compared with Mock#2 cells (Fig. 1C, middle). There were no differences in the expression levels of other NF-κB signaling pathway-related proteins (NIK, TAB1/2, TAK1, IKKα, and IKKy) between NDRG1/Cap43 and mock transfectants, and the expression levels of NF-κB subunits such as p65, p50, RelB, and p52 in Cap#11 and Cap#14 cells were similar to those in Mock#2 cells (Fig. 1C, right). Expression of IKKβ mRNA is slightly, but not significantly, decreased in NDRG1/Cap43 transfectants (Supplementary Fig. S2). In Cap#11 and Cap#14 cells, nuclear translocation of p65 was decreased by ~50% to 70% and that of p50 was decreased by ~80% compared with Mock#2, respectively (Fig. 1D, left). Expression of p65 and p50 showed only a slight increase in
cytosol fraction of Cap#11 and Cap#14 compared with that of Mock#2 (Fig. 1D, right).

We next performed EMSA to assess whether NDRG1/Cap43 altered the binding ability of NF-κB. One major shifted protein-DNA complex was observed after incubation of nuclear extracts prepared from Mock#2 cultured with 2% serum for 24 h (Fig. 2A). These complexes were specifically competed out with a 2-fold excess of the same unlabeled oligonucleotide but not with an unlabeled TRE and GC-box oligonucleotide. The protein-DNA complex after incubation of nuclear extracts was markedly decreased in Cap#11 and Cap#14 compared with Mock#2 when cultured with 2% serum. When protein-DNA complexes were incubated with antibodies against p65 and p50, supershifted bands were observed in Mock#2 (Fig. 2A, right).

We next examined whether the reduced level of p-IκBα could be restored by a potent inflammatory cytokine, TNF-α, in NDRG1/Cap43 transfecants (Fig. 2B). TNF-α induced phosphorylation of IκBα in both Cap#11 and Cap#14 at similar levels as their parental counterpart. However, cellular levels of IKKβ in Cap#11 and Cap#14 were not affected by TNF-α. Figure 2C shows that TNF-α also restored the expression of IL-8/CXCL8 in Cap#11 and Cap#14 cells to levels comparable with those in Mock#2 cells. Treatment with TNF-α also enhanced the affinity of p65 and p50 for NF-κB binding sites in Cap#11 and Cap#14 at similar levels to those in their parental counterparts (Fig. 2D). Taken together, NDRG1/Cap43 was not involved in TNF-α-induced NF-κB signaling pathway.
IKKβ overexpression overcomes NDRG1/Cap43-induced suppression of IκBα phosphorylation and chemokine expression. Expression of IKKβ was decreased in two NDRG1/Cap43 transfectants (Cap#11 and Cap#14). We examined whether exogenous IKKβ expression was able to restore the IκBα phosphorylation in NDRG1/Cap43 transfectants. Expression of IKKβ was augmented in both NDRG1/Cap43 and mock transfectants after transfection of the exogenous IKKβ gene (Fig. 3A). The phosphorylation of IκBα was increased in Cap#11 to a level comparable with that in Mock#2. Expression of Groα/CXCL1, ENA-78/CXCL5, and IL-8/CXCL8 was also significantly increased after transfection of IKKβ in Cap#11 cells when there was no apparent difference in the expression levels of these chemokines between empty and IKKβ transfection in Mock#2 (Fig. 3B). Expression of VEGF-A was also increased in IKKβ-transfected Cap#11 cells compared with that in empty-transfected Cap#11 cells. We observed that VEGF-A expression was decreased in IKKβ-transfected Mock#2 compared with empty-transfected Mock#2 cells, but the reason for this remains unclear.

IKKβ has been reported to hold a putative ubiquitin-like domain (20). We examined whether the reduced expression of IKKβ protein was restored by proteasome inhibitor, MG-132, in Cap#11 cells. MG-132 inhibited degradation of p-IκBα in both Mock#2 and Cap#11 cells (Fig. 3C). Furthermore, expression of IKKβ in Cap#11 cells was restored to similar levels as in Mock#2 cells when treated with MG-132. MG-132 did not significantly affect IKKβ mRNA expression in Mock#2 (P = 0.65) and Cap#11 (P = 0.48) cells.

A, EMSA using the NF-κB binding oligonucleotide. Nuclear extracts from three transfectants cultured in the presence of 2% serum were incubated with oligonucleotide as described in Materials and Methods. Black arrowheads, shifted bands; 0.08-, 0.4-, or 2-fold molar excess of unlabeled oligonucleotide was used as the competitor. A 2-fold molar excess of unlabeled oligonucleotide (TRE and GC-box) was used as a negative control for this competition assay. Arrows, positions of the supershifted bands (SS); NS, nonspecific band (white arrowheads). B, Western blot analysis of IκBα phosphorylation and expression of IKKα, IKKβ, and IKKγ in NDRG1/Cap43 and mock transfectants under serum-free conditions with or without TNF-α (20 ng/mL) stimulation for 30 min. C, ELISA assay analysis of IL-8/CXCL8 protein levels in NDRG1/Cap43 and mock transfectants of MIApaca-2 cells under serum-free conditions with or without TNF-α (20 ng/mL) for 24 h. Columns, mean of three independent experiments; bars, SE. *, P < 0.05; **, P < 0.01 versus mock transfectants. D, EMSA using the NF-κB binding oligonucleotide with nuclear extracts from three transfectants under serum-free conditions with or without TNF-α (20 ng/mL) for 30 min. Black arrowheads, shifted bands.
We further examined whether IKKβ was ubiquitinated or not in the presence of MG-132. As shown in Fig. 3D (bottom), ubiquitination of IKKβ was shown in Cap#11 cells, suggesting that a proteasomal degradation plays a role in down-regulation of IKKβ in the NDRG/Cap43-expressing cells.

NDRG1/Cap43 suppresses infiltration of inflammatory cells, expression of angiogenesis-related factors, tumor growth, and tumor angiogenesis. Consistent with our previous study (5), there was no difference in growth rates among Mock#2 and Cap#11 cells in culture (Fig. 4A). By contrast, tumor growth of Cap#11 was markedly reduced in comparison with Mock#2 in a subcutaneous mouse xenograft model (Fig. 4B, bottom). Immunoblotting analysis showed that NDRG1/Cap43 protein was consistently and highly expressed in Cap#11 tumors on day 49 after inoculation compared with Mock#2 tumors (Fig. 4B, top).

NDRG1/Cap43 was found to reduce the expression of chemokines and growth factors that function in chemotaxis of monocytes/macrophages and neutrophils (Fig. 1B; Supplementary Fig. S1). Mock#2 and Cap#11 tumor sections were further analyzed by immunohistochemistry for expression of microvessels (CD31), macrophages (F4/80), and neutrophils (Gr-1; Fig. 4C, top). MVD staining showed a markedly higher number of tumor neovessels in Mock#2 tumors than in Cap#11 tumors on day 49 after implantation (Fig. 4C, bottom). There appeared to be much lower infiltration of F4/80-positive macrophages and also Gr-1-positive infiltrating neutrophils in the stroma of Cap#11 tumors compared with that of Mock#2 tumors (Fig. 4C, bottom). NDRG1/Cap43 expression was thus closely associated with decreased MVD and also with a decreased number of infiltrating macrophages and neutrophils in mouse xenograft tumors. Expression of IL-8/CXCL8 and...
VEGF-A was significantly reduced in Cap#11 tumors compared with Mock#2 tumors (Fig. 4D), suggesting that reduced expression of such chemokines and growth factors was continuously maintained during tumor growth in this mouse xenograft model.

Association of NDRG1/Cap43 expression level with infiltrating inflammatory cells in tumors of pancreatic cancer patients. Expression of NDRG1/Cap43 was previously shown to be inversely correlated with MVD in the tumors of patients with pancreatic cancer (5). Based on the expression level of NDRG1/Cap43 in resected specimens from 37 patients with pancreatic ductal adenocarcinoma, we divided them into two groups: NDRG1/Cap43 positive (n = 18) and NDRG1/Cap43 negative (n = 19). Supplementary Table S3 shows the association between NDRG1/Cap43 expression and clinicopathologic variables such as age, gender, depth of invasion, lymph node metastasis, and pathologic stage in patients with pancreatic ductal adenocarcinoma. High NDRG1/Cap43 expression was significantly correlated with invasion depth (Supplementary Table S3).

In the human tumor stroma, some cases showed a lower number of infiltrating CD68+ macrophages/monocytes in NDRG1/Cap43-positive pancreatic cancer (Fig. 5A, a and b), whereas others showed a higher number of infiltrating CD68+ macrophages/monocytes in NDRG1/Cap43-negative pancreatic cancer (Fig. 5A, c and d). Quantitative analysis indicated that the number of infiltrating macrophages/monocytes was relatively higher in patients with NDRG1/Cap43-negative tumors than in those with NDRG1/Cap43-positive tumors (Fig. 5A, right), the mean number of infiltrating macrophages/monocytes being 97.5 and 62.3, respectively. However, similar numbers of infiltrating neutrophils were observed in the tumor stroma of patients with NDRG1/Cap43-positive and
NDRG1/Cap43-negative pancreatic cancer (Fig. 5B). Quantitative analysis showed that the mean number of infiltrating neutrophils was 40.2 in NDRG1/Cap43-negative specimens and 46.7 in NDRG1/Cap43-positive specimens, with no significant difference (Fig. 5B, right).

Our previous study showed that NDRG1/Cap43 expression levels were inversely correlated with MVD (5). Therefore, we further examined whether infiltration of macrophages/monocytes and neutrophils was associated with MVD in patients with NDRG1/Cap43-positive and NDRG1/Cap43-negative pancreatic cancer (n = 37). The number of infiltrating macrophages/monocytes was positively correlated with MVD (Fig. 5C; P < 0.05). However, there was no correlation between the number of infiltrating neutrophils and the MVD (Fig. 5D).

Discussion

We reported previously that NDRG1/Cap43 overexpression suppressed the expression of VEGF-A, IL-8/CXCL8, and matrix metalloproteinase-9 in pancreatic cancer cells (5). In the present study, we showed that NDRG1/Cap43 down-regulated the expression of several other genes, including chemotactants for inflammatory cells. We also observed that decreased expression of IL-8/CXCL8 and VEGF-A in mouse tumors was associated with high expression of NDRG1/Cap43. These chemotactants down-regulated by NDRG1/Cap43 had chemotactic effects on monocytes/macrophages and neutrophils. Our results showed that overexpression of NDRG1/Cap43 resulted in marked decrease in infiltration of macrophages and neutrophils in xenograft models.

One critical step in progression from a benign to a malignant state is angiogenesis. Infiltration of activated fibroblasts (21), macrophages/monocytes (22), and neutrophils (23) is expected to play a key role in the angiogenic switch of cancer (23–25). From our laboratory, we have also reported that infiltration of macrophages in the tumor stroma markedly promoted angiogenesis through the secretion of various proangiogenic cytokines and extracellular matrix-degrading proteases (18, 26–29). Groα/CXCL1, ENA-78/CXCL5, and IL-8/CXCL8 play an important role in tumor-associated angiogenesis and tumorigenesis in cancers of the kidney, pancreas, head and neck, and lung (30–33). Also, expression of CXC chemokines and VEGF-A would thus be expected to be closely involved in NDRG1/Cap43-induced suppression of tumor angiogenesis (Fig. 6). However, it is important to elucidate in more detail the underlying mechanism by which cytokines and growth factors are down-regulated in NDRG1/Cap43-positive cancer cells.
Factors are directly involved in the NDRG1/Cap43-dependent suppression of inflammatory cell infiltration and angiogenesis. Constitutive activation of NF-κB signaling pathway has been reported in many cancers, including pancreatic cancer (34). Fujioka and colleagues reported that pancreatic cancer cells expressing phosphorylation-defective IκBα showed decreased tumorigenicity in an orthotopic nude mouse model (35). In this mouse model, deletion of IκKβ in intestinal epithelial cells led to a decrease in tumor incidence without affecting tumor size (36). These studies suggested that the IκKβ–NF-κB signaling pathway plays an important role in tumor development.

In our present study, NDRG1/Cap43 reduced the expression of p-IκBα and its upstream regulator IκKβ in pancreatic cancer cells. However, we found no apparent phosphorylation of IκKα and IκKβ in NDRG1/Cap43 and mock transfectants under 2% serum condition (data not shown), suggesting that decreased expression of IκKβ is responsible for the loss of p-IκBα in NDRG1/Cap43 transfectants. The loss of p-IκBα results in reduction of both nuclear translocation of p65 and p50 and their binding to the NF-κB motif. NDRG1/Cap43-induced suppression of IκKβ was almost completely restored by a proteasome inhibitor. Introduction of an exogenous IκKβ gene was able to restore IκBα phosphorylation and expression of Groo/CXCL1, ENA-78/CXCL5, IL-8/CXCL8, and VEGF-A in NDRG1/Cap43 transfectants. In this study, we also found ubiquitination of IκKβ in NDRG1/Cap43 transfectant. A relevant study by May and colleagues showed that a ubiquitin-like domain of IκKβ is required for its functional activation (20). Taken together, the IκKβ–NF-κB pathway is expected to be attenuated by NDRG1/Cap43, resulting in decreased expression of angiogenesis and chemotaxis-related factors (see Fig. 6).

In various solid human tumors, an increase in the number of infiltrating tumor-associated macrophages has been shown to be closely associated with not only prognosis but also tumor angiogenesis (29, 37, 38). In our present study, a significantly decreased number of macrophages was also observed in clinical specimens of pancreatic cancer showing relatively higher NDRG1/Cap43 expression. The number of infiltrating macrophages was correlated with neovascularization in patients with pancreatic cancer. By contrast, we did not observe any significant difference in the number of infiltrating neutrophils between human pancreatic cancers showing low and high expression of NDRG1/Cap43. As shown in Fig. 4, in mouse xenograft models, chemotaxis of neutrophils and macrophages/monocytes was suppressed in Cap43 xenograft. By contrast, in clinical specimens of pancreatic cancer, infiltration of macrophages, but not neutrophils, was significantly associated with higher NDRG1/Cap43 expression. It remains unclear why NDRG1/Cap43 has no effect on the infiltration of neutrophils in clinical specimens of pancreatic cancer, and this question requires further study, focusing particularly on pancreatic cancer at earlier stages.

In conclusion, this study has shown that NDRG1/Cap43 decreases the expression of IκKβ and the NF-κB signaling pathway. As a consequence, NDRG1/Cap43 decreases the expression of chemoattractants such as CXC chemokines and VEGF-A for inflammatory cells, leading to a marked decrease in the recruitment of macrophages and/or neutrophils, along with angiogenesis suppression, in xenograft models. Therefore, NDRG1/Cap43 could be a potent biomarker for modulation of the tumor stroma in pancreatic cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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