Tumor Growth Suppression in Pancreatic Cancer by a Putative Metastasis Suppressor Gene Cap43/NDRG1/Drg-1 through Modulation of Angiogenesis

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Abstract

Cap43 has been identified as a nickel- and calcium-induced gene, and is also known as N-myc downstream-regulated gene 1 (NDRG1), Drg-1 and rit42. It is also reported that over-expression of Cap43 suppresses metastasis of some malignancies, but its precise role remains unclear. In this study, we asked how Cap43 could modulate the tumor growth of pancreatic cancer. Stable Cap43 cDNA transfectants of pancreatic cancer cells with Cap43 overexpression showed similar growth rates in culture as their control counterparts with low Cap43 protein level. By contrast, Cap43 over-expression showed a marked decrease in tumor growth rates in vivo. Moreover, a marked reduction in tumor-induced angiogenesis was observed. Gelatinolytic activity by matrix metalloproteinase-9 and invasion ability in Matrigel invasion activity were markedly decreased in pancreatic cancer cell lines with high Cap43 expression. Cellular expression of matrix metalloproteinase-9 and two major angiogenic factors, vascular endothelial growth factor and interleukin-8, were also significantly decreased in cell lines with Cap43 over-expression as compared with their parental counterparts. Immunohistochemical analysis of specimens from 65 patients with pancreatic ductal adenocarcinoma showed a significant association between Cap43 expression and tumor microvascular density (P = 0.0001) as well as depth of invasion (P = 0.0003), histopathologic grading (P = 0.0241), and overall survival rates for patients with pancreatic cancer (P = 0.0062). Thus, Cap43 could play a key role in the angiogenic on- or off-switch of tumor stroma in pancreatic ductal adenocarcinoma. (Cancer Res 2006; 66(12): 6233-42)

Introduction

The Cap43 gene has been identified as a nickel- and calcium-inducible gene (1), which is also identical to the described N-myc downstream-regulated gene 1 (NDRG1; ref. 2). This is one of the four closely related genes (NDRG1-4), the expression of which is down-regulated by c-myc or the N-myc/Max complex (2–5). Cap43 is also identical to the homocysteine-inducible gene, reduced in tumor cells (RTP/rit42; ref. 6), and to the differentiation-related gene-1 (Drg-1; ref. 7). The protein encoded by the Cap43 gene has a molecular weight of 43 kDa, has three unique 10–amino acid tandem-repeat sequences at its COOH terminus, and is phosphorylated by protein kinase A (8).

Although various characteristics of Cap43 have been reported by several laboratories, its exact function remains unclear. Cap43 gene expression is highly responsive to nickel, cobalt, oxidative stress, hypoxia, phorbol esters, vitamins A and D, steroids, histone deacetylase–targeting drugs, homocysteine, β-mercaptoethanol, tunicamycin, and lysophosphatidylcholine as well as oncogene (N-myc and c-myc), and tumor suppressor gene (p53 and VHL) products (1, 2, 6, 9, 10). Cap43 is expressed in most organs—particularly the prostate, ovary, colon, and kidney—and its expression is markedly modulated during postnatal development in the kidney, brain, liver, and gut (2, 3, 11, 12), suggesting a key role for this gene in organ maturation. A nonsense mutation of human Cap43 gene is causative for hereditary motor and sensory neuropathy-Lom (13). A relevant study by Okuda et al. established Cap43-deficient mice, and indicated that Cap43/NDRG1 is essential for the maintenance of the myelin sheaths in periphery nerves (14). Moreover, Cap43 expression was dramatically changed during the process of regeneration of periphery nerve system (15). Thus, Cap43 seems to play a key role in the development of the nervous system.

Concerning the plausible role of Cap43 in cancer progression, it was reported that overexpression of the Cap43 gene results in the inhibition of growth in colon cancer cells as well as suppression of metastasis in prostate, colon, and breast cancer cells (16–18). Cap43 gene expression is increased in many types of human cancers including colon, breast, prostate, kidney, liver, and brain cancers compared with normal tissue (19). By contrast, other groups have reported that expression of the Cap43 gene is up-regulated in normal cells and in highly differentiated cancer cells, whereas it is down-regulated in poorly differentiated cancer cells in colon and prostate cancers (16, 17). Thus, Cap43 seems to play a critical role in both differentiation of normal tissue and progression of cancer. In our laboratory, we identified the Cap43 gene as one of nine genes that were more highly expressed in cancerous compared with noncancerous regions of human renal tumors; we also observed high expression of Cap43 in macrophages infiltrating the stroma in renal cancer (20). Our previous study showed down-regulation of the Cap43 gene in renal cancer cells by the VHL tumor suppressor gene (10). However, it remains unclear whether Cap43 gene expression is associated with disease progression, prognosis, and malignant characteristics in human cancers other than prostate and breast cancer.

Pancreatic ductal adenocarcinoma is one of the most difficult neoplasms to treat curatively. Surgical resection is the standard
treatment for this neoplasm, however, even after surgery, the majority of patients die within the first year after diagnosis (21). Overall 5-year survival rates for pancreatic cancers are <25% after radical surgery (22). It is difficult to predict which patient is at risk of early relapse following surgery, or which patient with advanced stage disease will show good long-term survival. K-ras point mutations and inactivation of p53, p16, and SMAD4 are often associated with malignant characteristics in pancreatic cancers, but mutations of K-ras and p53 are not significantly associated with the prognosis of patients with pancreatic cancer (21, 23, 24). In the prognosis of patients with pancreatic cancer, there seems to be a significant correlation between microvessel density and the expression of vascular endothelial growth factor (VEGF) and PD-ECGF in the tumor, as well as with E-cadherin, p27, PEDF, and SMAD4 expression (24–27).

In this study, we investigated whether Cap43 expression could play any role in tumor growth and angiogenesis by pancreatic ductal adenocarcinoma. Moreover, we also asked if Cap43 could be associated with disease progression or the malignant properties of pancreatic ductal adenocarcinoma.

Materials and Methods

Materials and cell lines. Human pancreatic cancer cell lines were obtained as follows: BxPC-3, PAC-1, and MiaPaCa-2 were from the American Type Culture Collection (Manassas, VA); SUT-2 was from T. Iwamura at the Miyazaki Medical College, Miyazaki, Japan (28); and KP-1 and KP-4 were from A. Kono at the National Kyushu Cancer Center, Fukuoka, Japan (29). All cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and incubated in a humidified atmosphere of 5% CO2 at 37°C.

The rabbit polyclonal antibody against Cap43, which was raised by immunizing rabbits with a synthetic peptide corresponding to an internal sequence of human Cap43 coupled to keyhole limpet hemocyanin, was used as previously described (20). Other antibodies were purchased as follows: anti-β-actin antibody was from Abcam, Inc. (Cambridge, MA); anti-CD34 antibody was from PharMingen (San Diego, CA); and anti-CD3 antibody was from Nichirei (Tokyo, Japan).

Expression vector construction and transfection. Cap43 cDNA was amplified by reverse transcription-PCR (RT-PCR) using the 5’ and 3’ primers 5′-CATGTCTCCGGAGATGAGCAGATG-3’ and 5′-AGGCCGCTACGAGGAGACC-3’, respectively. Amplified Cap43 cDNA was ligated into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) and transferred to the pIREs-neo2 expression plasmid (pIREs-neo2-Cap43). Cells were transfected with pIREs-neo2-Cap43 or pIREs-neo2 using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Stable transfected clones were established using G418 selection.

Western blot analysis. Cells were rinsed with ice-cold PBS and lysed in buffer containing 50 mmol/L Tris-HCl, 350 mmol/L NaCl, 0.1% NP40, 5 mmol/L EDTA, 50 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mmol/L Na3VO4. Cell lysates were subjected to SDS-PAGE and blotted onto Immobilon membranes (Millipore Corp., Bedford, MA) as described previously (10). After transfer, the membrane was incubated with blocking solution followed by primary antibody. Antibody detection was done using an enhanced chemiluminescence system (Amersham Biosciences Corp., Piscataway, NJ; ref. 30). The intensity of the luminescence was quantified using a CCD camera combined with an image analysis system (LAS-1000; Fuji Film, Japan).

Intratumoral injection assay. Bx BioCoat Matrigel Invasion Chambers (BD Bioscience, Bedford, MA) were used according to the manufacturer’s instructions. Pancreatic cancer cells (1 × 106) in serum-free DMEM containing 0.1% bovine serum albumin were seeded onto Matrigel-coated filters in the upper chambers. In the lower chambers, DMEM containing 10% FBS was added as a chemoattractant. After 24 hours of incubation, cells on the upper surface of the filters were removed with a cotton swab, and the filters were fixed with 100% methanol and stained with Giemsa dye (31). The cells that had invaded to the lower side of the filters were viewed under an Olympus microscope and counted in five fields of view. The invasive ability of the cancer cells was expressed as the mean number of cells in five fields.

The assay was carried out as three independent experiments.

Gelatin zymography. Secreted metalloproteinases and their gelatinolytic activity were measured by zymography (32). Equal number of cells were plated on 24-well culture dishes and allowed to reach confluence over 24 hours. Cells were washed in PBS and incubated for 24 hours in 500 μL of serum-free DMEM. Conditioned media samples were then loaded onto 10% SDS-PAGE that had been copolymerized with 1 mg/mL of gelatin. Electrophoresis was done under nonreducing conditions. Gels were washed in 2.5% Triton X-100 for 30 minutes, and were incubated in collagenase buffer [100 mmol/L Tris-HCl (pH 8.0), 5 mmol/L CaCl2, 0.02% NaN3] for 40 hours at 37°C. Gels were stained for 30 minutes with 0.5% Coomassie blue and then destained (30% methanol and 10% acetic acid) thrice for 15 minutes. The presence of gelatinolytic activity due to secreted metalloproteinases was indicated by an unstained proteolytic region in the gel.

Quantitative real-time PCR analysis. Isolation of total cellular RNA was done as described previously (33). In brief, PCR amplification reaction mixtures (20 μL) contained cDNA, TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), primer pairs, and probe. The primer pairs and probe were obtained from Applied Biosystems. Thermal cycle conditions included holding the reactions at 50°C for 2 minutes and at 95°C for 10 minutes, and cycling for 40 cycles between 95°C for 15 seconds and 60°C for 1 minute. Results were collected and analyzed with an ABI Prism 7300 Sequence Detector System using the comparative ΔΔCt methods. All data were controlled for quantity of RNA input by performing measurements on an endogenous reference gene (GAPDH).

Animals. All animal experiments were approved by the Committee on the Ethics of Animal Experiments in Kyushu University Graduate School of Medical Sciences, Japan. Male BALB/C mice (6-10 weeks old) were purchased from Kyudo Co., Ltd. (Tosu, Japan). Male athymic nude mice 5 weeks of age, weighing 20 to 22 g, and specific pathogen-free, were obtained from Charles River Laboratories (Yokohama, Japan). Mice were housed in microisolator cages with 12-hour light/dark cycles. 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.

Mouse dorsal air sac assay. This assay was carried out with 6- to 10-week-old male mice as previously described (34, 35). Cells (3 × 106) were suspended in 150 μL of DMEM containing 2% FBS, and was injected into a chamber, which consisted of a ring covered with Millipore filters (0.45 μm pore size, Millipore Corp.) on each side. This was implanted into an air sac produced by injecting 10 mL of air s.c. on the back of an anesthetized mouse (50 mg/kg pentobarbital, i.p.) on day 0. On day 5, the chambers were removed from the s.c. fascia, and replaced with black rings of the same inner diameter as the chambers. Photographs of these sites were assessed by counting the number of newly formed vessels >3 mm in length within the area of the rings. The number of newly formed blood vessels were each scored on scales from 0 to 8. Results are expressed as mean ± SD.

Nude mouse xenograft models and determination of microvascular density. Cells were suspended in sterile PBS at a concentration of 106 cells/mL and 100 μL were injected s.c. into the right flank of the nude mice. Tumor size was measured by calipers in the largest diameter and at a perpendicular dimension in order to calculate the tumor area.

Tumors of nude mouse xenograft. Intratumoral microvessels were detected using a rat anti-mouse CD31 described previously (33). Tumors in nude mice were removed, snap-frozen in optimum cutting temperature compound (SAKURA Finetechical, Japan), and 6-μm 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 CD31 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 the CD34 antigen.
In all samples, the mean value for the number of microvessels was calculated from four vascular hotspots. Only the 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.

**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 ELISA kits (31). Cells were plated in 24-well dishes in medium containing 10% FBS. When the cells reached subconfluence, the medium was replaced with DMEM containing 2% FBS, then the cells were incubated for 24 hours. Results were normalized for the number of cells and reported as picograms of growth factor/10^5 cells/24 hours. Tumors obtained from mice were homogenized in T-PER Tissue Protein Extraction reagent containing protein inhibitor cocktail (Pierce, Rockford, IL) and centrifuged at 13,000 g for 10 minutes. The concentrations of VEGF and IL-8 in the supernatant of the lysates were measured by using an ELISA kit following the manufacturer's protocols.

**Patients and specimens.** Surgically resected specimens from 65 patients with pancreatic ductal adenocarcinomas were studied. All patients underwent surgical resection between 1991 and 1998 in the Department of Surgery at the Kurume University Hospital, Japan. Among these patients, 43 underwent pancreaticoduodenectomy, 21 underwent distal pancreatectomy, and one underwent total pancreatectomy. All subjects underwent extended radical lymphadenectomy. Pancreatic resection was not done in patients presenting with distant site metastasis. All cases of mucinous cystadenocarcinoma or intraductal papillary-mucinous carcinoma were excluded from our study. Pancreatic ductal adenocarcinoma tissues were obtained from 37 men and 28 women with a mean age of 64 years (age range, 40-80 years).

**Immunohistochemical analysis of Cap43.** All human specimens were fixed in 10% formalin and embedded in paraffin wax. Unstained 4-μm sections were then cut from paraffin blocks for immunohistochemical analysis. The sections were stained with rabbit anti-Cap43 polyclonal antibody (10, 20). Cap43-specific immunoreactivity was scored by estimating the percentage of labeled tumor cells as follows: score 0, no positive cells; score +, <30% positive cancer cells; score ++, 30% to 80% positive cancer cells; and score +++, >80% positive cancer cells. Specimens were considered positive for Cap43 expression when the score was ++ or +++, and were considered negative for Cap43 expression when the score was 0 or +. All procedures were done by two independent assessors and one pathologist, all of whom had no previous knowledge of the clinical outcome for this series of cases. Individual specimens with discordant results among the investigators were re-evaluated.

**Statistical analysis.** Data are expressed as mean ± SD. Comparisons between groups were done using Welch's t test. The Kaplan-Meier method was used to calculate the overall survival rate and the prognostic significance was evaluated by the log-rank test. The correlation of Cap43 immunoreactivity with the patients' clinicopathologic variables were analyzed by Fisher's exact test. Differences were considered significant at P < 0.05.
Results

Cap43 levels had no effects on the cell proliferation rates of pancreatic cancer cells in culture. We first compared the protein levels of Cap43 in six pancreatic cancer cell lines. Of the six lines, KP-1, BxPC-3, and SUIT-2 showed relatively higher Cap43 expression, whereas KP-4, PANC-1, and MIApaca-2 showed relatively lower Cap43 expression (Fig. 1A). We next established cell lines that express higher amounts of Cap43 by transfection of Cap43 cDNA into two cell lines, PANC-1 and MIApaca-2. Two Cap43 cDNA transfectants from PANC-1, PANC/Cap-6, and PANC/Cap-7, and those from MIApaca-2, paca/Cap-11, and paca/Cap-14, showed enhanced expression of Cap43 as compared with their parental mock transfectants, PANC/Mock-3 and paca/Mock-2, respectively (Fig. 1B). We also compared the growth rates of mock and Cap43 cDNA transfectants of PANC-1 and MIApaca-2 in the presence of 10% serum (Fig. 1C). No significant difference in growth rates was observed between their parental counterparts and transfected cell lines.

A marked decrease of tumor growth and angiogenesis by Cap43 overexpression in pancreatic cancer cells. We next examined whether overexpression of Cap43 could modulate tumor growth in mice under xenograft assay systems. The tumor growth of both paca/Cap-11 and paca/Cap-14 showed markedly reduced rates as compared with their mock-transfected line, paca/Mock-2 (Fig. 2A and B). We could not observe any tumor growth when PANC/Mock-3, PANC/Cap-6, and PANC/Cap-7 were transplanted. Determination of MVD showed much greater development of neovessels in paca/Mock-2 tumor than both paca/Cap-11 and paca/Cap-14 tumors (Fig. 2C). Quantitative analysis showed a significant decreasing number of MVD in both Cap43 transfectants by >50% of the control counterparts (Fig. 2D).

We compared the tumor-induced angiogenic activity of Cap43 cDNA transfectants and their mock transfectants in PANC-1 and MIApaca-2. Dorsal air sac assay showed the apparent development of new microvessels (in, arrowhead, Fig. 3) with curled structures and many tiny bleeding spots by PANC/Mock-3 and paca/Mock-2, but only a slight, if any, development of such microvessels by their Cap43 cDNA transfectants (Fig. 3A). Quantitative analysis showed that the development of neovessels by both Cap43 cDNA
transfectants was <20% of their control counterparts in both MIApaca-2 and PANC-1 (Fig. 3B).

**Effect of Cap43 on invasive ability in pancreatic cancer cells.**

We next examined if the expression of Cap43 could affect the cellular locomotion/invasion activity of pancreatic cancer cell lines by Matrigel invasion assay. Of the six cell lines, BxPC-3 and SUIT-2 showed relatively low invasive activity in comparison with the other four cell lines in photographs of Matrigel-coated filters (data not shown). Quantitative analysis showed a markedly lower invasive ability for two cell lines, BxPC-3 and SUIT-2, which expressed relatively high levels of Cap43 (see Fig. 1A), and intermediate invasiveness for one cell line, KP-1, which expressed a moderate Cap43 level (Fig. 3C). By contrast, three cell lines (KP-4, PANC-1, and MIApaca-2), which expressed relatively low Cap43 levels, showed higher invasive abilities. We further compared the invasive ability of Cap43 cDNA transfectants and their mock transfectants in PANC-1 and MIApaca-2. Two Cap43 transfectants consistently showed markedly decreased invasive ability compared with the mock transfectants in both PANC-1 and MIApaca-2 (Fig. 3D).

**A marked effect of Cap43 on the expression of matrix metalloproteinase-9, VEGF, and IL-8.** To determine why angiogenesis and cell locomotion/invasion are strongly affected by Cap43 expression, we compared the expression levels of angiogenesis-related factors, matrix metalloproteinases (MMP), VEGF, and IL-8 between Cap43 cDNA transfectants and their control counterparts. The expression of MMPs in cancer cells as well as vascular endothelial cells, regulate neovascularization by enhancement of pericellular fibrinolysis and cellular locomotion (36). Of the many MMPs, MMP-9 plays a critical role in the angiogenic switch during carcinogenesis (37). By using conventional gelatin zymography, we compared the activity of MMPs between Cap43-transfected lines and their control counterparts in PANC-1 and MIApaca-2. Two Cap43 transfectants, PANC/Cap-6 and PANC/Cap-7, which express high amounts of Cap43 protein showed much reduced MMP-9 activity (gelatinase B) compared to their mock transfectants.
with their parental counterparts (PANC-1/Mock-3), whereas PANC/Cap-6 and -7 showed similar MMP-2 activities as its parental counterpart (Fig. 4A). We could not observe any apparent activity of MMP-2 and MMP-9 in cell lines derived from MIpaca-2 in this assay (data not shown). Both Cap3-overexpressing cell lines of PANC-1 showed <10% MMP-9 mRNA levels of that of PANC/Mock-3 (Fig. 4B). Although the mRNA levels of MMP-9 were ~10% in MIpaca-2 as compared with that in PANC-1, reduced levels of MMP-9 mRNA were also observed in Cap3-overexpressing cell lines of MIpaca-2 in comparison with their mock transfectant (Fig. 4B).

We also compared the cellular production of two potent angiogenic factors, VEGF and IL-8, by ELISA assay and reverse transcription-PCR between Cap3 cDNA transfectants and their control counterparts. Figure 4C showed a marked decrease in the production of VEGF in both PANC/Cap-6 and PANC/Cap-7 in comparison with PANC/Mock-3. However, we could not observe any apparent production of IL-8 in these lines derived from PANC-1, and also that of VEGF in cell lines derived from MIpaca-2, assayed by ELISA (data not shown). In contrast, cellular levels of IL-8 were ~10% and 60% in paca/Cap-11 and paca/Cap-14, respectively, of those in paca/Mock-2 (Fig. 4C). Determination of mRNA levels by quantitative real-time PCR also showed the marked decrease in cellular mRNA levels of both VEGF and IL-8 in Cap3 cDNA transfectants as compared with their parental counterparts in both PANC-1 and MIpaca-2 (Fig. 4D).

We further compared the production of VEGF and IL-8 in the s.c. tumors under in vivo conditions. Determination of mRNA levels by quantitative real-time PCR showed 12- and 18-fold increases in Cap3 mRNA levels in paca/Cap-14 tumor 1 and tumor 2, respectively, over those in paca/Mock-2 tumor when tumors were removed at 8 weeks after inoculation. We determined the expression levels of VEGF and IL-8 in tumors by ELISA assay system (see Materials and Methods). VEGF expression levels (pg/mg protein) were not detectable in both paca/Cap-14 tumor 1 and 2 when those were 280 ± 8.9 in paca/Mock-2 tumor. IL-8 expression levels (pg/mg protein) were 142 ± 10.2 and 46 ± 8.4 in paca/Cap-14 tumor 1 and tumor 2, respectively, when those were 580 ± 82.4 in paca/Mock-2 tumor.

Association of Cap3 expression with angiogenesis and clinicopathologic characteristics as well as prognosis in patients with pancreatic cancer. The expression of immunoreactive Cap3 was examined immunohistochemically in resected specimens of 65 patients with pancreatic ductal adenocarcinoma. Two clinical examples of positive immunostaining (a and b) and two examples of negative immunostaining (c and d) are presented in Fig. 5A. Based on this immunohistochemical staining for Cap3, we classified the 65 cancer specimens into positive (n = 27) and negative (n = 38) groups. We further investigated whether Cap3 expression in patients with pancreatic ductal adenocarcinoma was associated with clinicopathologic variables such as age, gender, depth of invasion, lymph node metastasis, pathologic stage, and...
histopathologic grading. Cap43 expression was significantly correlated with depth of invasion ($P = 0.0003$), pathologic stage ($P = 0.0263$), and histopathologic grading ($P = 0.0244$), but not with lymph node metastasis (Table 1). We also determined MVD in tumors by immunostaining analysis with anti-CD34 antibody. Two clinical samples of Cap43-positive immunostaining with lower MVD (scoring number, <40; a-d) and two clinical samples of Cap43-negative specimens (e and g) are presented in Fig. 5B. Based on this immunohistochemical analysis, we classified patients into high (scoring number, >40; $n = 33$) and low (scoring number, <40; $n = 32$) MVD groups. High MVD was found to be significantly associated with negative Cap43 expression, whereas low MVD was significantly associated with positive Cap43 expression ($P = 0.0001$; Table 1). The median values of MVD in Cap43-positive specimens was 28.3, and those of MVD in Cap43-negative specimens was 48.8 (Fig. 5C). Immunohistochemical analysis of clinical specimens therefore suggested that high Cap43 expression was closely associated with low angiogenic status of pancreatic ductal adenocarcinoma.

Finally, we investigated whether Cap43 expression was associated with the prognosis for patients with pancreatic ductal adenocarcinoma. Kaplan-Meier analysis indicated that Cap43 protein expression showed a statistically significant correlation with survival after radical surgery ($P = 0.0062$; Fig. 5D), with cases lacking Cap43 expression having unfavorable prognoses. The median survival time for patients with tumors that were positive for Cap43 expression was 24.7 months, compared with 10.9 months for patients with tumors negative for Cap43 expression.

Discussion
Overexpression of Cap43 did not alter the growth rates of human prostate and colon cancer cells (16, 17). Consistent with these studies, cellular proliferation assay of Cap43-overexpressing cell lines of MIaPaca-2 and PANC-1 showed similar growth rates as their parental counterparts in culture. Tumor growth in vivo showed a marked decrease by Cap43 overexpression in MIaPaca-2 when their parental counterparts showed high tumor growth rates. Thus, Cap43 overexpression seemed to specifically affect tumor

Figure 5. Correlation between Cap43 expression and MVD. Immunohistochemical analysis of Cap43 and microvessels in pancreatic ductal adenocarcinoma with anti-Cap43 and CD34 antibody (A and B). A, two clinical samples of patients with pancreatic ductal adenocarcinoma who had good long-term survival after surgery showing Cap43 expression (a and b). Two clinical samples of patients with pancreatic ductal adenocarcinoma who had poor survival after surgery showing reduced Cap43 expression (c and d). Original magnification, ×100. B, ductal adenocarcinoma cells in Cap43 positive specimens (a and c), and in Cap43-negative specimens (e and g). Immunohistochemical staining of anti-CD34 antibody. A tumor area with low vessel density (b and d) and high vessel density (f and h) are shown. Original magnification, ×100. C, correlation between Cap43 expression and MVD. The median value of MVD in a Cap43-negative specimen was 48.8 and in a Cap43-positive specimen was 28.3. D, Kaplan-Meier analysis of overall survival according to Cap43 expression levels in pancreatic ductal adenocarcinoma. The survival of patients whose cancer cells did not express Cap43 was significantly ($P = 0.0062$) worse than that of patients whose cancer cells expressed Cap43. Patients with Cap43-positive cancer ($n = 27$) and those with reduced expression of Cap43 ($n = 38$).
Cap43 in cancer cells might alter the tumor stroma microenvironment, resulting in a block of tumor progression. Consistent with this notion, our present study further showed a marked decrease in angiogenesis by Cap43 overexpression in MIApaca-2 or PANC-1 in the dorsal air sac assay, and also decreased MVD in tumor induced by Cap43 overexpression in MIApaca-2 as compared with their parental counterparts. Cellular production of angiogenesis-related factors such as MMP-9, VEGF, and IL-8 were markedly reduced in cell lines with Cap43 overproduction. In gelatin zymography, there seemed to be almost no MMP-9 activity in PANC/Cap-6 and PANC/Cap-7 when their parental counterpart had MMP-9 activity. Although there was no apparent activity corresponding with MMP-9 and MMP-2 in cell lines of MIApaca-2, MMP-9 mRNA level was also reduced in both Cap43 transfectants of MIApaca-2. In the Matrigel invasion assay, pancreatic cancer cell lines with relatively higher Cap43 expression showed lower invasive ability. Cap43 overexpression may reduce both the expression and activity of MMP-9, resulting in altered cellular locomotion and invasive ability. A relevant study by Bergers et al. has reported that MMP-9 triggers the angiogenic switch during carcinogenesis of pancreas in transgenic mice (37). In this study, genetic ablation of MMP-9, but not that of MMP-2, impairs the induction of angiogenesis. Moreover, expression of two representative angiogenic factors, VEGF and IL-8, was also markedly reduced in cell lines overexpressing Cap43. Cellular production of VEGF and IL-8 was also inversely correlated with Cap43 levels. VEGF and IL-8 production was markedly reduced in cell lines overexpressing Cap43. Cellular production of VEGF and IL-8 was also inversely correlated with Cap43 transfectants of MIApaca-2. In the Matrigel invasion assay, pancreatic cancer cell lines with relatively higher Cap43 expression showed lower invasive ability. Cap43 overexpression may reduce both the expression and activity of MMP-9, resulting in altered cellular locomotion and invasive ability. A relevant study by Bergers et al. has reported that MMP-9 triggers the angiogenic switch during carcinogenesis of pancreas in transgenic mice (37). In this study, genetic ablation of MMP-9, but not that of MMP-2, impairs the induction of angiogenesis. Moreover, expression of two representative angiogenic factors, VEGF and IL-8, was also markedly reduced in cell lines overexpressing Cap43. Cellular production of VEGF and IL-8 was also inversely correlated with Cap43 levels. VEGF and IL-8 production was markedly decreased in the subcutaneous tumor by Cap43 gene transfectant in comparison with tumors of the parental counterpart, suggesting that expression of VEGF and IL-8 is affected in both in vitro and in vivo conditions by Cap43 in pancreatic cancer. The low tumor angiogenic activity by Cap43 overexpression could be due to decreased expression of angiogenic factors such as VEGF, IL-8, and MMP-9. A more precise study should be required to understand the underlying mechanism of how the expression of such angiogenesis-related factors could be modulated by Cap43.

Consistent with previous studies (22, 40, 41), we found that the survival rates for 65 patients with pancreatic cancer were significantly correlated with histopathologic grading and depth of invasion. We further observed a close association between low Cap43 expression and poor prognosis in pancreatic ductal adenocarcinoma. Cap43 was originally isolated as a differentiation-related gene and is mostly expressed in differentiated epithelial cells (9, 11), and this might be relevant to its association with the histopathologic grading of pancreatic cancer. To our surprise, high MVD in clinical specimens of pancreatic cancer was found to be significantly associated with low Cap43 expression levels (Table 1; Fig. 5), consistent with our experimental animal model for tumor growth and MVD. On the other hand, poor prognosis and malignant progression are often significantly associated with VEGF and PD-ECGF, as well as with MVD in tumors (see Introduction). From our present study, MVD in clinical specimens of pancreatic cancer was also significantly associated not only with Cap43 (see Table 1) but also with prognosis of patients.6 Pancreatic tumor growth is regulated by positive and negative angiogenesis modulators (42), and kinase inhibitors of both VEGF and EGF receptors were found to reduce the tumor

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### Table 1. Association between Cap43 expression and clinicopathologic variables in 65 patients with pancreatic ductal adenocarcinoma

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<th>Variable</th>
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<tr>
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<td>I</td>
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<td>7</td>
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</tr>
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<td>II</td>
<td>4</td>
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<tr>
<td>III</td>
<td>30</td>
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<tr>
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<tr>
<td>Histopathologic grading</td>
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<tr>
<td>G1</td>
<td>28</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>G2, G3</td>
<td>37</td>
<td>11</td>
<td>26</td>
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<tr>
<td>Microvascular density</td>
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<td>Low (&lt;40)</td>
<td>32</td>
<td>21</td>
<td>11</td>
</tr>
<tr>
<td>High (&gt;40)</td>
<td>33</td>
<td>6</td>
<td>27</td>
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</table>

*Analyses were carried out using Fisher's exact test.

P< 0.05 considered statistically different.

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growth in vivo but not cell growth in vitro, suggesting the critical role of Cap43 in the modulation of tumor stroma in pancreatic cancer. Previous studies using animal experimental models have shown that Cap43 overexpression suppresses metastasis by prostate and colon cancer cells, suggesting that Cap43/NDRG1 is a metastasis suppressor gene (16, 17). A relevant study by Bandyopadhyay et al. have reported that the Cap43/Drg-1 protein was significantly reduced in breast cancer cells, particularly, in patients with lymph node or bone metastasis as compared to those with localized breast cancer (18). Cap43 could thus affect not only metastasis/invasion but also tumor growth, suggesting that Cap43/NDRG1 could be a putative metastasis suppressor gene. Concerning the possible mechanism for up-regulation of Cap43, Le and Richardson have previously reported that treatment of breast cancer cells by iron chelator with high antiproliferation activity up-regulates the expression of Cap43/NDRG1 gene through hypoxia-inducible factor-1α-dependent and -independent pathways (38). Our recent study showed estradiol induced down-regulation of Cap43 gene in estradiol receptor-positive breast cancer cell lines, and also that Cap43 expression was inversely correlated with the expression of estradiol receptor-α but not with progesterone receptor in human breast cancer (39). Thus, expression of Cap43 in breast cancer seems to be greatly affected under various conditions including hypoxia, metals, and estradiol.
growth and metastasis of pancreatic cancers in a xenograft model (43). Clinical studies also showed a close association of angiogenesis and/or enhanced expression of VEGF and other angiogenic factors with disease progression and patient survival in pancreatic cancers (44–46). Shi et al. have reported that IL-8 expression renders pancreatic cancer cells more tumorigenic and metastatic under various microenvironmental changes including hypoxia and acidosis (47). On the other hand, infiltration of tumor-associated macrophages is closely associated with angiogenesis in various human malignancies (48, 49), and our previous study showed the expression of Cap43 in tumor-associated macrophages in human renal cancers (20). We also observed the expression of Cap43 in macrophages as well as cancer cells in pancreatic cancers when clinical specimens were examined by immunohistochemical analysis (data not shown). The role of Cap43 in macrophages, however, remains to be further studied.

In conclusion, we first demonstrated that Cap43 could switch-off angiogenesis and cell locomotion/invasion by pancreatic cancer cells. A putative metastasis suppressor function of Cap43/NDRG1 might be due to such modulation of angiogenesis in tumor stroma. Cap43 could be a novel target for angiogenesis and malignant characteristics of pancreatic cancers. How Cap43 could control the expression of angiogenesis-related genes in pancreatic cancer cells is now in progress in our laboratory.

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References


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