

**Figure 6.** Effects of C-RAF depletion or sorafenib on cyclin E expression in NSCLC cells with mutant *KRAS*. Cells harboring mutant *KRAS* were transiently transfected for 48 h with nonspecific (control), B-RAF, or C-RAF siRNAs or were exposed to 15  $\mu$ mol/L sorafenib for 24 h in complete medium. Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to B-RAF, C-RAF, cyclin E, and  $\beta$ -actin.

proteins, our present data suggest that the observed down-regulation of cyclin E may contribute to the G<sub>1</sub> arrest induced by C-RAF depletion or by sorafenib in NSCLC cells with mutant *KRAS*.

Sorafenib inhibits several RTKs that participate in neovascularization, including vascular endothelial growth factor receptor (VEGFR)-2 and VEGFR-3 (16). Inhibition of angiogenesis might thus be expected to contribute to the inhibition of tumor growth by this drug in addition to its effects on RAF signaling. Although sorafenib was previously shown to inhibit the growth of a variety of human tumor xenografts in mice (13, 16, 46), it has been difficult to measure the relative contributions of its antiangiogenic activity and its direct antitumor activity mediated by RAF inhibition. In the present study, we have provided insight into the inhibitory effect of sorafenib on tumor cell growth *in vitro* that is mediated by inhibition of RAF signaling pathways. Our results suggest that sorafenib targets B-RAF in NSCLC cells with wild-type *KRAS* and C-RAF in those with mutant *KRAS*, and they provide a rationale for future clinical investigation of the therapeutic efficacy of sorafenib for NSCLC patients.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## A phase-II trial of dose-dense chemotherapy in patients with disseminated thymoma: report of a Japan Clinical Oncology Group trial (JCOG 9605)

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**BACKGROUND:** To evaluate the safety and efficacy of dose-dense weekly chemotherapy in the treatment of advanced thymoma. **METHODS:** Subjects comprised patients with histologically documented chemotherapy-naïve thymoma with stage-IVa or IVb disease. Thymic carcinoma, carcinosarcoma or lymphoma cases were excluded. Patients received 9 weeks of chemotherapy: cisplatin (25 mg m<sup>-2</sup>) on weeks 1–9; vincristine (1 mg m<sup>-2</sup>) on weeks 1, 2, 4, 6 and 8; and doxorubicin (40 mg m<sup>-2</sup>) and etoposide (80 mg m<sup>-2</sup>) on days 1–3 of weeks 1, 3, 5, 7 and 9. Chemotherapy courses were supported by granulocyte colony-stimulating factor. Post-protocol local therapy was allowed.

**RESULTS:** From July 1997 to March 2004, 30 patients were entered. Three were ineligible due to different histology. Chemotherapy-associated toxicity was mainly haematological and was well tolerated, with no deaths due to toxicity, and 87% of patients completed the planned 9-week regimen. Overall response rate was 59%, with 16 of the 27 eligible patients achieving partial response. Median progression-free survival (PFS) was 0.79 years (95% confidence interval: 0.52–1.40 years), and PFS at 1 and 2 years was 37 and 15%, respectively. Overall survival rates at 2 and 5 years were 89 and 65%, respectively.

**CONCLUSION:** In stage-IV thymoma patients, weekly dose-dense chemotherapy offers similar activity to conventional regimens.

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Thymoma is a rare thoracic tumour, but remains one of the most common tumours originating in the mediastinum (Thomas *et al*, 1999; Giaccone, 2005; Girard *et al*, 2009). Clinical behaviour tends to be indolent, but dissemination into the pleural space eventually occurs and sometimes distant metastasis arise (Thomas *et al*, 1999). Thymoma is frequently associated with paraneoplastic syndromes such as myasthenia gravis or pure red cell aplasia (Thomas *et al*, 1999; Giaccone, 2005). No International Union Against Cancer (UICC) TNM classification is available, and the Masaoka classification has been widely used for clinical staging (Masaoka *et al*, 1981; Girard *et al*, 2009).

The majority of thymomas are discovered at a limited stage, representing Masaoka stage-I or II, and surgical resection is the treatment of choice for such cases (Thomas *et al*, 1999; Giaccone, 2005; Girard *et al*, 2009). Even when the tumour invades neighbouring organs, as stage-III disease, surgical resection with postoperative radiotherapy is the preferred treatment when complete resection can be achieved (Curran *et al*, 1988; Urgesi *et al*, 1990; Ogawa *et al*, 2002; Strobel *et al*, 2004).

Systemic chemotherapy is usually used for stage-IVa (with pleural or pericardial dissemination) or stage-IVb disease (with lymphogenous or haematogenous metastases), but optimal management is less well established (Thomas *et al*, 1999; Girard *et al*, 2009). Several reports have described favourable outcomes in limited numbers of patients with stage-IVa disease treated using multimodal treatment including surgery (Kim *et al*, 2004; Yokoi *et al*, 2007).

Conversely, thymomas are generally reported to be chemotherapy-sensitive tumours, with response rates of 50–70% to

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combination chemotherapy (Fornasiero *et al*, 1990; Loehrer *et al*, 1994, 1997, 2001; Giaccone *et al*, 1996; Berruti *et al*, 1999; Kim *et al*, 2004; Lucchi *et al*, 2006; Yokoi *et al*, 2007). Active agents include cisplatin (CDDP), vincristine (VCR), doxorubicin (ADM), etoposide (ETP), cyclophosphamide (CPM) and ifosfamide (IFX). Recent reports have shown marginal activity of pemetrexed (Loehrer *et al*, 2006) and combined carboplatin and paclitaxel (Lemma *et al*, 2008).

Dose-dense chemotherapy with the CODE combination (CDDP-VCR-ADM-ETP) and addition of granulocyte colony-stimulating factor (G-CSF) can be safely administered to patients with advanced lung cancer (Murray *et al*, 1991; Fukuoka *et al*, 1997). Theoretically, this approach might be suitable for chemosensitive tumours such as small-cell lung cancer and thymoma (Goldie and Coldman, 1983, 1984; Levin and Hryniuk, 1987; Murray, 1987). Because some pilot data in Japan suggested that administration of 12 weeks of the CODE chemotherapy was barely feasible, subsequent Japanese trials used a modified schedule, which was shortened to 9 weeks (Fukuoka *et al*, 1997; Furuse *et al*, 1998).

In 1996, the Japan Clinical Oncology Group (JCOG) initiated two clinical trials for advanced thymoma: one aimed at evaluating the safety and efficacy of the CODE regimen in stage IV, disseminated thymoma (JCOG 9605), and the other aimed at evaluating the safety and efficacy of CODE combination chemotherapy followed by surgical resection and postoperative radiotherapy in initially unresectable stage-III thymoma (JCOG 9606). The primary end-point in each study was progression-free survival (PFS). The results of JCOG 9605 are reported herein.

## PATIENTS AND METHODS

### Eligibility criteria

Patients with chemotherapy-naïve, histologically documented thymoma at Masaoka stage IVa or IVb were eligible for entry into the study. Thymoma must have been confirmed histologically and thymic tumours with other histology, such as thymic carcinoma, carcinoid or lymphoma, were excluded. Each patient was required to fulfil the following criteria: age, 15–70 years; Eastern Cooperative Oncology Group (ECOG) performance status (PS), 0–2; adequate organ function, that is, leukocyte count  $\geq 4000 \mu\text{l}^{-1}$ , platelet count  $\geq 10^5 \mu\text{l}^{-1}$ , hemoglobin  $\geq 10.0 \text{ g dl}^{-1}$ , serum creatinine  $< 1.5 \text{ mg dl}^{-1}$ , creatinine clearance  $\geq 60 \text{ ml min}^{-1}$ , serum bilirubin  $< 1.5 \text{ mg dl}^{-1}$ , serum alanine transaminase and aspartate transaminase levels less than double the upper limit of the institutional normal range; and  $\text{PaO}_2 \geq 70 \text{ mm Hg}$ . Exclusion criteria included uncontrolled heart disease, uncontrolled diabetes or hypertension, pulmonary fibrosis or active pneumonitis as evidenced on chest radiography, infections necessitating systemic use of antibiotics, disease necessitating emergency radiotherapy such as superior vena cava obstruction syndrome, active concomitant malignancy and women who were pregnant or lactating. Also excluded were those patients with grave complications of thymoma, such as pure red cell aplasia or hypogammaglobulinemia. Myasthenia gravis was allowed and these patients were not excluded *per se*.

Patient eligibility was confirmed by the JCOG Data Center before patient registration. This study protocol was approved by the institutional review board at each participating centre and written informed consent was obtained from all patients prior to enrolment.

### Treatment Plan

**Chemotherapy** Patients received the 9-week CODE combination chemotherapy as described below. Each chemotherapeutic agent was administered intravenously.

Week 1: CDDP  $25 \text{ mg m}^{-2}$  on day 1 with antiemetics and ample hydration; VCR ( $1 \text{ mg m}^{-2}$ ) on day 1; ADM ( $40 \text{ mg m}^{-2}$ ) on day 1 and ETP ( $80 \text{ mg m}^{-2}$ ) on days 1–3.

Weeks 2, 4, 6 and 8: CDDP ( $25 \text{ mg m}^{-2}$ ) on day 1 with antiemetics and ample hydration and VCR ( $1 \text{ mg m}^{-2}$ ) on day 1.

Weeks 3, 5, 7 and 9: CDDP ( $25 \text{ mg m}^{-2}$ ) on day 1 with antiemetics and ample hydration, ADM ( $40 \text{ mg m}^{-2}$ ) on day 1 and ETP ( $80 \text{ mg m}^{-2}$ ) on days 1–3.

Each week, G-CSF (filgrastim ( $50 \mu\text{g m}^{-2} \text{ day}^{-1}$ ) or lenograstim ( $2 \mu\text{g kg}^{-1} \text{ day}^{-1}$ )) was administered by subcutaneous injection, except on days when chemotherapy was administered or when leukocyte count was  $\geq 10\,000 \mu\text{l}^{-1}$ . Corticosteroid was used only as part of the antiemetic regimen, and the specific drug and dosage were not regulated by the protocol.

Dose and schedule modifications were performed as follows: when leukocyte count decreased to  $< 2,000 \mu\text{l}^{-1}$  or platelet count decreased to  $< 50\,000 \mu\text{l}^{-1}$ , chemotherapy was delayed by 1 week. If PS decreased to 3–4 or temperature reached  $\geq 38.0^\circ\text{C}$ , therapy was likewise delayed for 1 week. No dose modification of chemotherapy drugs was adopted for toxicity.

### Post-protocol therapy

Surgery or radiotherapy was allowed after the completion of chemotherapy, at the discretion of the attending physician, even in the absence of apparent tumour regrowth. Conversely, additional chemotherapy without evidence of disease progression was not allowed.

Post-treatment after disease progression was not limited by the study protocol.

### Patient evaluation and follow-up

Before enrolment into the study, each patient underwent complete medical history taking and physical examination (including neurological check-up for signs of myasthenia gravis), determination of blood cell counts, serum biochemistry testing, arterial blood gas analysis, pulmonary function testing, electrocardiography, chest radiography, computed tomography (CT) of the chest, CT or ultrasonography of the upper abdomen, whole-brain CT or magnetic resonance imaging (MRI) and an isotope bone scan. Blood-cell counts, serum biochemistry testing and chest radiography were performed weekly during each course of chemotherapy.

The toxicity of chemotherapy was evaluated according to the JCOG Toxicity Criteria (Tobinai *et al*, 1993), modified from version 1 of the National Cancer Institute Common Toxicity Criteria (NCI-CTC). Tumour responses were assessed radiographically according to the standard, two-dimensional WHO criteria (Miller *et al*, 1981), and were classified as complete response (CR), partial response (PR), no change (NC), progressive disease (PD) or non-evaluable (NE). After completion of the protocol therapy, patients were followed up with periodic re-evaluation, including chest CT every 6 months for the first 2 years and annually thereafter.

### Central review

Radiographic reviews for the eligibility of enrolled patients and clinical responses were performed at the time of the study group meeting, held every 3–4 months. The study coordinator (H Kunitoh) and a few selected investigators from the group reviewed the radiographic films. The clinical response data presented below were all confirmed by this central review. Reviews of pathological specimens were not performed, because of insufficient logistics of the study group at the time of the study activation in 1997.

## Endpoints and statistical considerations

The primary endpoint in each study was PFS. Due the rarity of the tumour and the accrual reported in US trials, which required 10 years to register 26 patients with locally advanced (stage-III) disease (Loehrer *et al*, 1997) and 9 years for 31 patients with disseminated (stage-IV) disease (Loehrer *et al*, 1994), we presumed we would be capable of accruing 30 patients in the target accrual period of 4 years. The sample size was, therefore, not determined based on statistical calculations. The expected PFS for the JCOG 9605 study was 2 years, which would give a 95% confidence interval of 1.3–3.0 years with 30 cases.

The initial study design thus envisioned enrolment of 30 fully eligible cases over 3 years for the study, with a follow-up period of 2 years.

Secondary endpoints included toxicity and safety, objective tumour response to chemotherapy, pattern of relapse, and overall survival (OS).

Progression-free survival and OS were calculated from the date of enrolment and estimated using the Kaplan–Meier method. Progression-free survival was censored at the last date verifiable as progression-free, and OS was censored as of the date of last follow-up. During the accrual period, an interim analysis for futility was planned after half of the patients had been registered and followed for  $\geq 3$  months. All analyses were performed using SAS software version 8.2/9.1 (SAS Institute, Cary, NC, USA).

## RESULTS

### Patient characteristics

A total of 30 patients from seven institutions were enrolled from July 1997 to March 2004. Three patients were later found ineligible due to wrong histology, with two cases of thymic carcinoma and one case of carcinoid. These mistakes occurred due to technical problems in the patient registry. Since the ineligible cases did receive the protocol therapy, all 30 patients were analysed for characteristics and toxicity. Twenty-seven eligible patients were analysed for clinical response and survival (PFS and OS). Patient characteristics are shown in Table 1.

### Chemotherapy delivery and toxicity

Nine weeks of chemotherapy were performed for 26 of the original 30 patients (87%). The other four patients included one patient receiving 7 weeks, two receiving 6 weeks and one receiving 3 weeks of therapy. Median duration of chemotherapy for the 26 patients who underwent the planned nine cycles was 10 weeks (range, 9–12 weeks).

Table 2 summarises the major toxicities of chemotherapy, which were mainly haematological. Although 70% of patients experienced grade-IV neutropenia, this was generally transient and rarely complicated by infection/fever. Overall, toxicities were well tolerated and no deaths due to toxicity occurred.

### Other and late complications

Four patients showed thymoma-related complications. One patient suffered from myasthenia gravis crisis occurring during chemotherapy, but subsequently recovered. Another patient showed newly diagnosed myasthenia gravis 2.5 years after completion of the protocol therapy, and thymectomy and resection of the residual tumour were performed. Two other cases had pure red cell aplasia occurring later in the clinical course with disease progression of the thymomas.

**Table 1** Patient characteristics

| Item                                |            |
|-------------------------------------|------------|
| Sex                                 |            |
| Male/female                         | 16/14      |
| Age (years)                         |            |
| Median/range                        | 47.5/29–69 |
| ECOG performance status             |            |
| PS0/PS1/PS2                         | 11/18/1    |
| Masaoka stage                       |            |
| IVa/IVb                             | 22/8       |
| Smoking history                     |            |
| No                                  | 9          |
| Yes (median pack–years)             | 21 (22)    |
| Myasthenia gravis                   |            |
| No/yes                              | 28/2       |
| Histology: thymoma and eligible     | 27         |
| Lymphocyte predominance             | 12         |
| Mixed cell                          | 9          |
| Epithelioid cell                    | 4          |
| Clear cell                          | 1          |
| Spindle cell                        | 0          |
| Unclassified                        | 1          |
| Histology: not thymoma (ineligible) | 3          |
| Carcinoma                           | 2          |
| Carcinoid                           | 1          |
| Lymphoma                            | 0          |
| Prior therapy                       |            |
| None                                | 26         |
| Surgery                             | 2          |
| Surgery and radiation               | 2          |

Abbreviations: ECOG = Eastern Cooperative Oncology Group; PS = performance status.

**Table 2** Toxicity of chemotherapy (n = 30)

| Toxicity         | Grades 1/2 | Grade 3 | Grade 4 | %Grade 3/4 |
|------------------|------------|---------|---------|------------|
| Leukopenia       | 3/6        | 12      | 8       | 67         |
| Neutropenia      | 3/1        | 5       | 21      | 87         |
| Anemia           | 0/5        | 25      | ND      | 83         |
| Thrombocytopenia | 4/6        | 5       | 3       | 27         |
| ALT              | 9/0        | 0       | 0       | 0          |
| Creatinine       | 2/1        | 0       | 0       | 0          |
| PaO <sub>2</sub> | 9/2        | 0       | 0       | 0          |
| Emesis           | 13/11      | 2       | ND      | 7          |
| Diarrhoea        | 4/2        | 0       | 0       | 0          |
| Stomatitis       | 4/3        | 0       | 0       | 0          |
| Constipation     | 3/4        | 2       | 0       | 7          |
| Neuropathy       | 11/2       | 0       | ND      | 0          |
| Infection        | 3/4        | 3       | 0       | 10         |

Abbreviations: ALT = alanine transaminase; ND = not defined (the JCOG toxicity criteria did not define grade IV in these toxicities).

### Clinical response to chemotherapy

Clinical responses of the 27 eligible patients to chemotherapy were judged radiologically and confirmed by central review. Responses were as follows: CR, 0 patients; PR, 16 patients; NC, 10 patients and PD, 1 patient. Overall response rate was 59% (95% confidence interval, 39–78%).

**Post-protocol therapy**

Post-protocol local therapy was administered to 18 of the 27 eligible patients (67%). Eight patients (all with stage-IVa disease) underwent surgical resection and 13 patients (nine with stage-IVa disease and four with stage-IVb disease) received thoracic radiotherapy, with three patients receiving both. Whether patients received local therapy after disease progression was not recorded on case report forms.

After disease progression, 16 of the 27 patients (59%) received additional chemotherapy. Post-protocol chemotherapy included platinum re-challenge, irinotecan, taxanes and investigational agents. Clinical response data to those therapies are not available.

**PFS and OS**

Survival data were finally updated in March 2006, 2 years after accrual of the last patient. Figure 1 shows PFS and OS curves of the 27 eligible patients. Median PFS was 0.79 years (95% confidence interval, 0.52–1.40 years) and PFS at 1 and 2 years was 37 and 15%, respectively. Median OS was 6.1 years and OS at 2 and 5 years was 89 and 65%, respectively.

Overall survival was longer for stage-IVa patients than for stage-IVb patients (Figure 2, median, 6.8 years and 3.5 years, respectively), but PFS was similar (Figure 3, median, 0.79 years for IVa patients and 0.78 years for IVb patients).

**Pattern of relapse**

As of the data cut-off, 26 of the 27 eligible patients had experienced tumour relapse. Sites of initial relapse comprised the primary site only in seven cases (27%), pleural or pericardial dissemination in seven cases (27%) and primary site and pleural/pericardial dissemination in nine cases (35%). Thus, 23 of the 26 patients with relapse initially showed regrowth of the primary and/or pleural or pericardial dissemination, with only three patients (12%) showing initial relapse at distant organs.

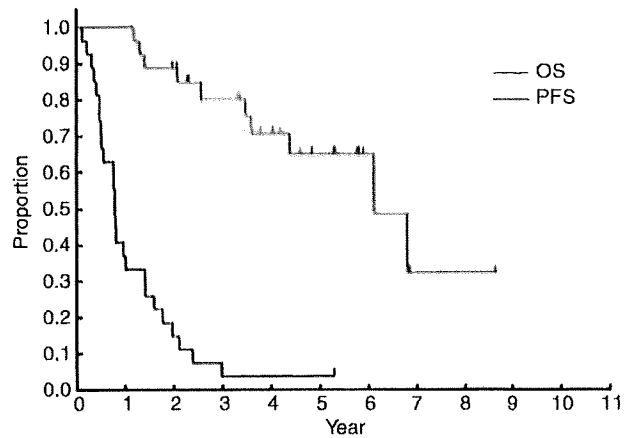
**DISCUSSION**

Few prospective trials of chemotherapy have been described for patients with advanced thymoma. Most prior studies have combined stage-III, localised disease and stage-IV, disseminated disease (Table 3). In addition, most have also included both thymoma and thymic carcinoma histology.

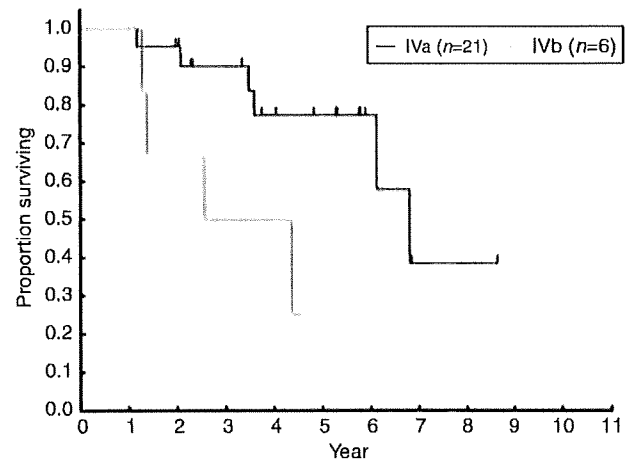
We have reported results for patients with stage-IV disease, for which systemic therapy should be the first choice. Among previous studies, only those from the ECOG separately reported results for stage-III and stage-IV patients (Loehrer *et al*, 1994, 1997). The ECOG took 9 years to accrue 31 patients with stage-IV disease, including patients with thymic carcinoma (Loehrer *et al*, 1994). We prospectively accrued patients with thymoma only and excluded thymic carcinoma, as thymoma and thymic carcinoma clearly differ in clinical presentation and prognosis, and trials involving these pathologies should, thus, be reported separately (Eng *et al*, 2004; Giaccone, 2005; Lemma *et al*, 2008).

Trials of systemic chemotherapy for thymoma have reported response rates of 50–90%, so this tumour is generally considered sensitive to chemotherapy (Thomas *et al*, 1999). Dose-dense chemotherapy such as the CODE four-drug combination has been argued to be theoretically suitable for the treatment of such chemosensitive tumours (Murray, 1987).

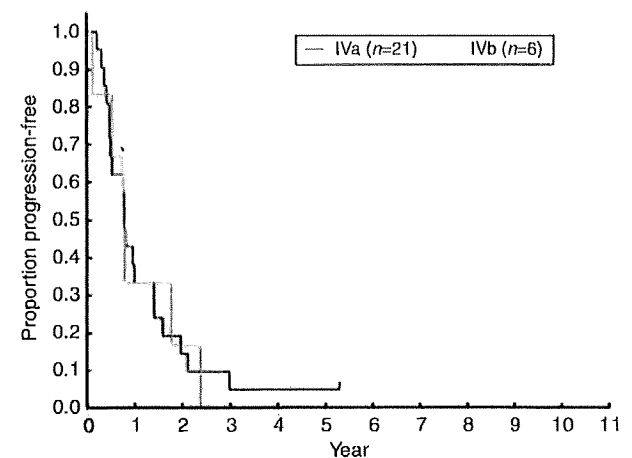
Although our results showed that dose-dense CODE chemotherapy could be safely administered to thymoma patients, efficacy was not remarkable. The overall response rate was about 60%, no different from prior reports employing conventional-dose chemotherapy (Table 3). Progression-free survival was 9 months, falling far short of the expected 2 years. Although OS studies



**Figure 1** Progression-free survival and OS of the 27 eligible patients.



**Figure 2** Overall survival according to Masaoka stage (stage IVa vs IVb).



**Figure 3** Progression-free survival according to Masaoka stage (stage IVa vs IVb).

compared favourably with the corresponding ECOG trial (Loehrer *et al*, 1994), attempting to reach a valid conclusion would be difficult due to the small sample sizes. In addition, OS could be

Clinical Studies

**Table 3** Reports of combination chemotherapy for thymoma

| Regimen                                      | Stage  | Patients <sup>a</sup> | ORR | Reference               |
|--|--------|-----------------------|-----|-------------------------|
| <i>Anthracycline-containing regimens</i>     |        |                       |     |                         |
| ADOC (S)                                     | III/IV | 32                    | 91% | Fornasiero et al (1990) |
| PAC (G)                                      | IV     | 30                    | 50% | Loehrer et al (1994)    |
| PAC (G)                                      | III    | 23                    | 70% | Loehrer et al (1997)    |
| ADOC (S)                                     | III/IV | 16                    | 81% | Beruti et al (1999)     |
| PAC (G)                                      | III/IV | 22                    | 77% | Kim et al (2004)        |
| PAE (S)                                      | III/IV | 30                    | 73% | Lucchi et al (2006)     |
| CAMP (S)                                     | III/IV | 14                    | 93% | Yokoi et al (2007)      |
| CODE (G)                                     | IV     | 27                    | 59% | Current study           |
| <i>Non-anthracycline-containing regimens</i> |        |                       |     |                         |
| PE (G)                                       | III/IV | 16                    | 56% | Giaccone et al (1996)   |
| VIP (G)                                      | III/IV | 20                    | 35% | Loehrer et al (1997)    |
| CP (G)                                       | III/IV | 23                    | 35% | Lemma et al (2008)      |

Abbreviations: ADOC = doxorubicin, cisplatin, vincristine, cyclophosphamide; CAMP = cisplatin, doxorubicin, methylprednisolone; CODE = cisplatin, vincristine, doxorubicin, etoposide; CP = carboplatin, paclitaxel; G = prospective multicenter group trial; ORR = overall response rate; PAC = cisplatin, doxorubicin, cyclophosphamide; PAE = cisplatin, epidoxorubicin, etoposide; PE = cisplatin, etoposide; S = single-center experience; VIP = etoposide, ifosfamide, cisplatin. <sup>a</sup>Number of assessable patients.

greatly affected by post-study local therapy especially in patients with stage-IVa disease, as combined therapy trial including stage-IVa patients suggested (Kim et al, 2004). In fact, this might be one reason why OS of stage-IVa patients was much longer than that of stage-IVb patients, whereas PFS was similar.

It could be argued that shortened CODE chemotherapy, used in Japan due to feasibility problem, led to inadequate results due to insufficient total dosages of chemotherapy drugs. However, another intensive chemotherapy, ETP-IFX-CDDP (VIP) supported by G-CSF, has also reported disappointingly low response rates and no better survival (Loehrer et al, 2001). Hanna et al (2001) reported five patients with prior chemotherapy treated with high-dose chemotherapy and stem cell support, but concluded that no superiority to conventional therapy was evident. Taken together with our results, intensification of chemotherapy does not appear sufficiently promising for treating advanced thymoma.

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Appendix 1

STUDY PARTICIPANTS

The following institutions and investigators participated in the trial:

Asahikawa Medical College (Yoshinobu Osaki), National Cancer Center Hospital East (Yutaka Nishiwaki, Kaoru Kubota, Nagahiro Saijo), National Cancer Center Hospital (Tomohide Tamura, Noboru Yamamoto, Hideo Kunitoh), Kanagawa Cancer Center (Kazumasa Noda, Fumihiko Oshita), Niigata Cancer Center

Hospital (Akira Yokoyama, Yuko Tsukada), Kinki University Hospital (Kazuhiko Nakagawa, Isamu Okamoto) and Osaka City General Hospital (Koji Takeda, Haruko Daga).

Appendix 2

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## N-myc Downstream Regulated Gene 1/Cap43 Suppresses Tumor Growth and Angiogenesis of Pancreatic Cancer through Attenuation of Inhibitor of $\kappa$ B Kinase $\beta$ 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 chemoattractants, 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- $\kappa$ B (NF- $\kappa$ B) signaling attenuation through marked decreases in inhibitor of  $\kappa$ B kinase (IKK)  $\beta$  expression and I $\kappa$ B $\alpha$  phosphorylation. Decreased IKK $\beta$  expression in cells overexpressing NDRG1/Cap43 resulted in reduction of both nuclear translocation of p65 and p50 and their binding to the NF- $\kappa$ B motif. The introduction of an exogenous IKK $\beta$  gene restored NDRG1/Cap43-suppressed expression of melanoma growth-stimulating activity  $\alpha$ /CXCL1, epithelial-derived neutrophil activating protein-78/CXCL5, interleukin-8/CXCL8 and vascular endothelial growth factor-A, accompanied by increased phosphorylation of I $\kappa$ B $\alpha$  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- $\kappa$ B 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 in patients 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- $\kappa$ B (NF- $\kappa$ B) 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- $\beta$ -actin antibody (Abcam); anti-NIK, anti-TAB1/2, anti-TAK1, anti-inhibitor of  $\kappa$ B kinase (IKK)  $\alpha$ , anti-IKK $\beta$ , anti-IKK $\gamma$ , anti-p-I $\kappa$ B $\alpha$ , anti-p65, 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- $\alpha$  (TNF- $\alpha$ ) and MG-132 was purchased from R&D Systems and Calbiochem.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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**Plasmid constructs.** To obtain full-length cDNA of human IKK $\beta$ , PCR was carried out on a SuperScript cDNA library (Invitrogen) using the following primer pairs: 5'-ATGAGCTGGTCACCTTCCCTGACAAC-3' and 5'-TCATGAGGCTGCTCCAGGCAGCTG-3' (IKK $\beta$ ). The PCR product was ligated into the pGEM-T easy vector (Promega), and Flag-IKK $\beta$  was ligated into the p3xFLAG-CMV10 vector (Sigma).

**Oligonucleotide microarray analysis.** Duplicate samples were prepared for microarray hybridization. Total RNA (2  $\mu$ g) was reverse transcribed using a GeneChip 3'-Amplification Regents 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 Scanner3000, and the image was analyzed using GeneChip Operating Software version 1 as described previously (15).

**Determination of melanoma growth-stimulating activity  $\alpha$ /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  $\alpha$  (Gro $\alpha$ )/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  $\mu$ g) was incubated for 15 min at room temperature with a  $1 \times 10^4$  counts/min  $^{32}$ P-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(deoxyinosinic-deoxycytidylic 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  $\mu$ mol/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 Na $_2$ VO $_4$ ] supplemented with a mixture of protease inhibitors. Lysates were incubated with anti-ubiquitin antibody for 2 h at 4°C and 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-IKK $\beta$  antibody as described previously (17). The intensity of the luminescence was quantified using a CCD camera combined with an image analysis system (LAS-1000; 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 ( $1.0 \times 10^7$  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  $\pm$  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).

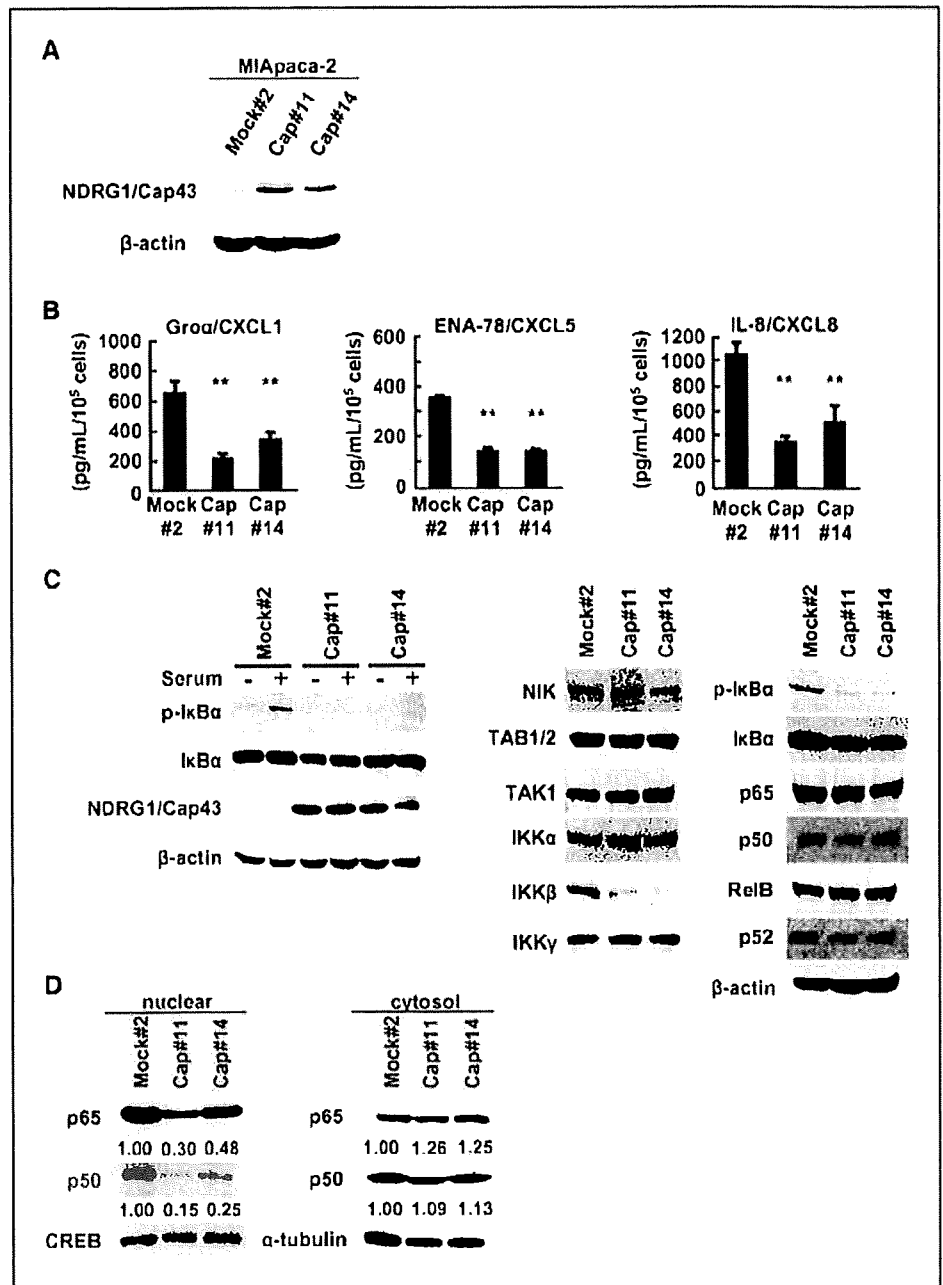
We selected eight genes predicted to be associated with adhesion, growth, and chemotaxis (Supplementary Table S2). Because our previous study showed that NDRG1/Cap43 overexpression in pancreatic cancer cells reduced the expression of angiogenesis-related factors such as VEGF-A and IL-8/CXCL8 (5), we also selected these genes, the expression of which showed a decrease of  $\sim$ 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 NCAM1, which was up-regulated by NDRG1/Cap43, as a control. The mRNA expression levels of Gro $\alpha$ /CXCL1, ENA-78/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 $\alpha$ /CXCL1 and ENA-78/CXCL5 as well as IL-8/CXCL8.

**NDRG1/Cap43 suppresses the NF- $\kappa$ B signaling pathway in pancreatic cancer cells.** Representative angiogenic factors such as IL-8/CXCL8 and VEGF-A are regulated by NF- $\kappa$ B (19). We investigated whether NDRG1/Cap43 expression interfered with the NF- $\kappa$ B signaling pathway in pancreatic cancer cells. The phosphorylation of I $\kappa$ B $\alpha$  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 I $\kappa$ B $\alpha$  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- $\kappa$ B signaling pathway to examine which molecules are responsible for the difference in the phosphorylation level of I $\kappa$ B $\alpha$  between NDRG1/Cap43 and mock transfectants. Phosphorylation of I $\kappa$ B $\alpha$  is regulated by the IKK complex, which consists of two catalytic subunits, IKK $\alpha$  and IKK $\beta$ , and a regulatory component, IKK $\gamma$ /NEMO. The expression of IKK $\beta$  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- $\kappa$ B signaling pathway-related proteins (NIK, TAB1/2, TAK1, IKK $\alpha$ , and IKK $\gamma$ ) between NDRG1/Cap43 and mock transfectants, and the expression levels of NF- $\kappa$ B 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 IKK $\beta$  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  $\sim$ 50% to 70% and that of p50 was decreased by  $\sim$ 80% compared with Mock#2, respectively (Fig. 1D, left). Expression of p65 and p50 showed only a slight increase in

**Figure 1.** NDRG1/Cap43 reduces expression levels of CXC chemokines and phosphorylation of I $\kappa$ B $\alpha$  in MIApaca-2 cell lines showing low and high NDRG1/Cap43 expression. All experiments, except A, were done with 2% serum for 24 h. A, Western blot analysis of NDRG1/Cap43 expression in MIApaca-2 transfectants. B, ELISA assay analysis of Gro $\alpha$ /CXCL1, ENA-78/CXCL5, and IL-8/CXCL8 protein levels in NDRG1/Cap43 and mock transfectants of MIApaca-2 cells. Columns, mean of three independent experiments; bars, SE. \*\*,  $P < 0.01$  versus Mock#2 cells. C, phosphorylation of I $\kappa$ B $\alpha$  and NDRG1/Cap43 expression in NDRG1/Cap43 and mock transfectants cultured with or without 2% serum for 24 h was measured by Western blotting (left). Western blot analysis of the expression of NF- $\kappa$ B signaling pathway-related proteins using whole-cell lysates prepared from NDRG1/Cap43 and mock transfectants (middle and right). D, Western blot analysis of the expression of p65 and p50 in nuclear (left) and cytosol (right) extracts prepared from NDRG1/Cap43 and mock transfectants. Levels of protein expression are expressed relative to the level of p65 or p50 protein in Mock#2, which is presented as 1.00.



cytosol fraction of Cap#11 and Cap#14 compared with that of Mock#2 (Fig. 1D, right).

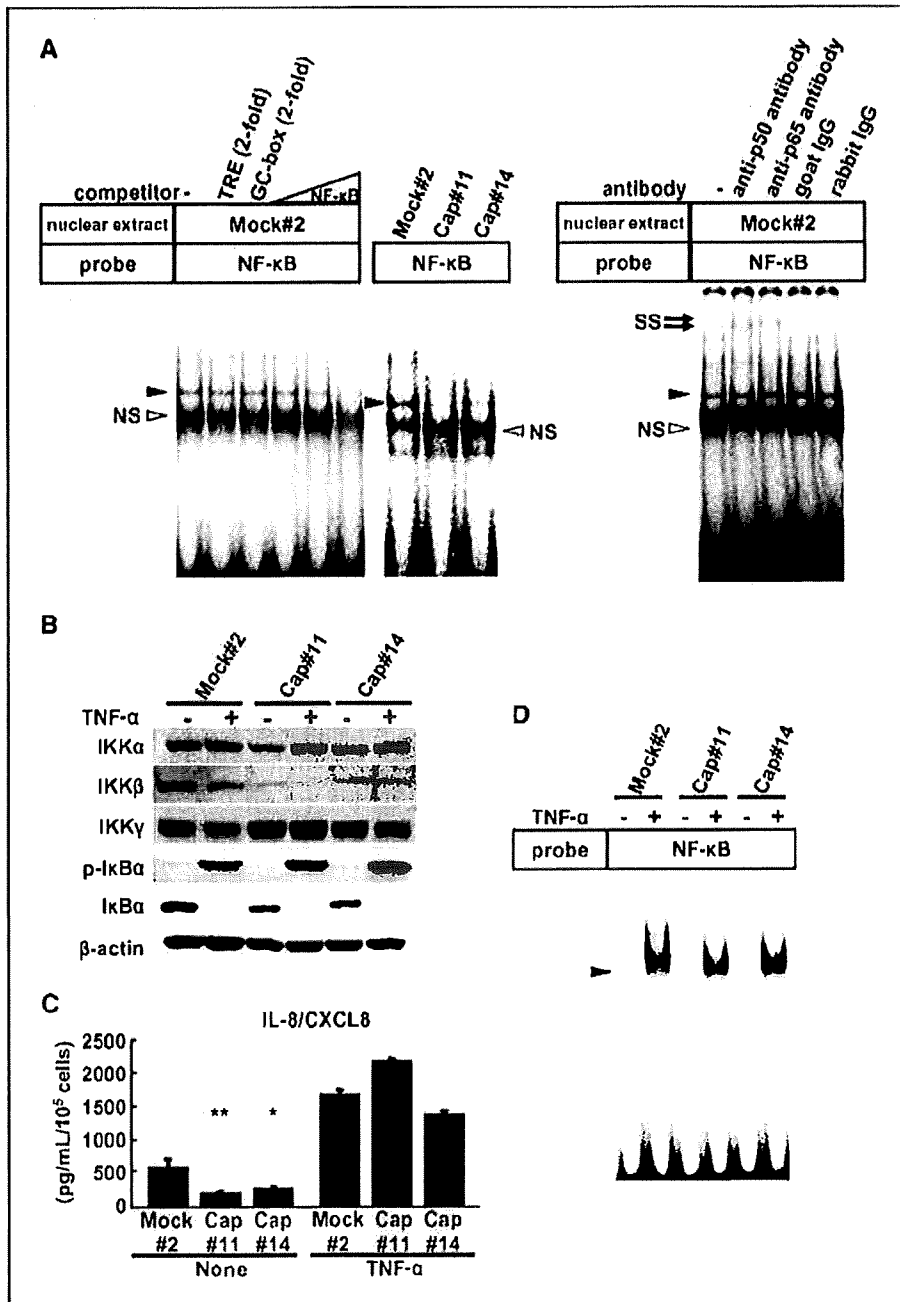
We next performed EMSA to assess whether NDRG1/Cap43 altered the binding ability of NF- $\kappa$ B. One major shifted protein-DNA complex was observed after incubation of nuclear extracts prepared from Mock#2 cultured with 2% serum for 24 h (Fig. 2A). These complexes were specifically competed out with 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). We next examined whether the reduced level of p-I $\kappa$ B $\alpha$  could be restored by a potent inflammatory cytokine, TNF- $\alpha$ , in NDRG1/Cap43 transfectants (Fig. 2B). TNF- $\alpha$  induced phosphorylation of I $\kappa$ B $\alpha$  in both Cap#11 and Cap#14 at similar levels as their parental counterpart. However, cellular levels of IKK $\beta$  in Cap#11 and Cap#14 were not affected by TNF- $\alpha$ . Figure 2C shows that TNF- $\alpha$  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- $\alpha$  also enhanced the affinity of p65 and p50 for NF- $\kappa$ B 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- $\alpha$ -induced NF- $\kappa$ B signaling pathway.

**IKK $\beta$  overexpression overcomes NDRG1/Cap43-induced suppression of I $\kappa$ B $\alpha$  phosphorylation and chemokine expression.** Expression of IKK $\beta$  was decreased in two NDRG1/Cap43 transfectants (Cap#11 and Cap#14). We examined whether exogenous IKK $\beta$  expression was able to restore the I $\kappa$ B $\alpha$  phosphorylation in NDRG1/Cap43 transfectants. Expression of IKK $\beta$  was augmented in both NDRG1/Cap43 and mock transfectants after transfection of the exogenous IKK $\beta$  gene (Fig. 3A). The phosphorylation of I $\kappa$ B $\alpha$  was increased in Cap#11 to a level comparable with that in Mock#2. Expression of Gro $\alpha$ /CXCL1, ENA-78/CXCL5, and IL-8/CXCL8 was also significantly increased after transfection of IKK $\beta$  in Cap#11 cells when there was no apparent difference in the expression levels of these chemokines between empty and IKK $\beta$  transfection in

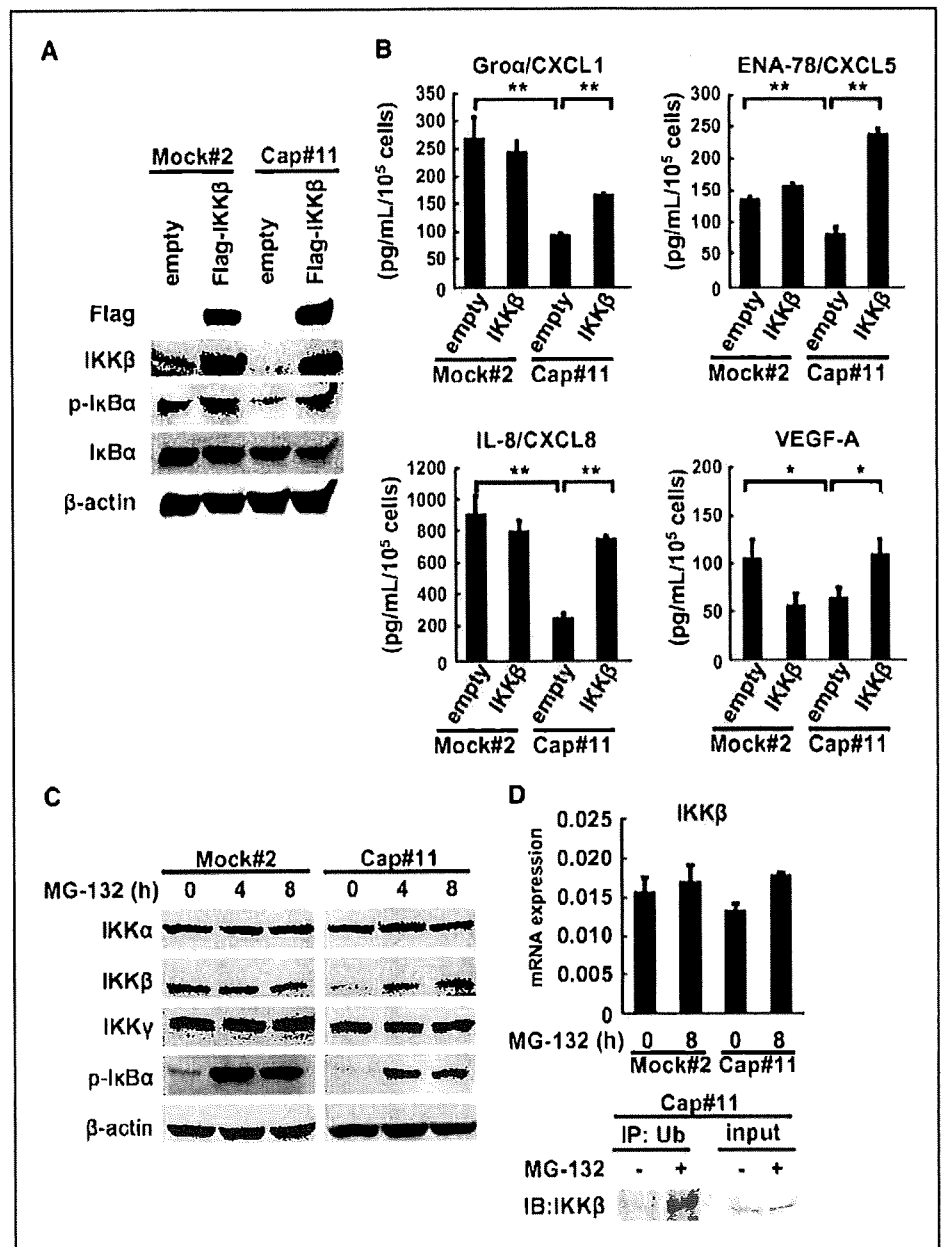
Mock#2 (Fig. 3B). Expression of VEGF-A was also increased in IKK $\beta$ -transfected Cap#11 cells compared with that in empty-transfected Cap#11 cells. We observed that VEGF-A expression was decreased in IKK $\beta$ -transfected Mock#2 compared with empty-transfected Mock#2 cells, but the reason for this remains unclear.

IKK $\beta$  has been reported to hold a putative ubiquitin-like domain (20). We examined whether the reduced expression of IKK $\beta$  protein was restored by proteasome inhibitor, MG-132, in Cap#11 cells. MG-132 inhibited degradation of p-I $\kappa$ B $\alpha$  in both Mock#2 and Cap#11 cells (Fig. 3C). Furthermore, expression of IKK $\beta$  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 IKK $\beta$  mRNA expression in Mock#2 ( $P = 0.65$ ) and Cap#11 ( $P = 0.48$ ) cells



**Figure 2.** Suppression of binding activity of NF- $\kappa$ B by NDRG1/Cap43. **A**, EMSA using the NF- $\kappa$ B 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 I $\kappa$ B $\alpha$  phosphorylation and expression of IKK $\alpha$ , IKK $\beta$ , and IKK $\gamma$  in NDRG1/Cap43 and mock transfectants under serum-free conditions with or without TNF- $\alpha$  (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- $\alpha$  (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- $\kappa$ B binding oligonucleotide with nuclear extracts from three transfectants under serum-free conditions with or without TNF- $\alpha$  (20 ng/mL) for 30 min. Black arrowheads, shifted bands.

**Figure 3.** IKK $\beta$  overexpression restores the level of p-IkBa and expression of the CXC chemokines and VEGF-A. **A**, expression of the exogenous IKK $\beta$  gene and phosphorylation of IkBa in MIApaca-2 transfectants. NDRG1/Cap43 and mock transfectants were transiently transfected with the IKK $\beta$  expression vector. At 30 h after IKK $\beta$  transfection, the cells were again seeded and cultured for 18 h and then cultured in medium containing 2% serum for 24 h. **B**, ELISA assay analysis of Groa/CXCL1, ENA-78/CXCL5, IL-8/CXCL8, and VEGF-A protein levels in Mock#2 and Cap#11 that were transfected with the IKK $\beta$  expression vector. At 48 h after IKK $\beta$  transfection, the cells were cultured in medium containing 2% serum for 24 h. Columns, mean of three independent experiments; bars, SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus empty-transfected Cap#11 cells. **C**, Western blot analysis of IkBa phosphorylation and IKK $\alpha$ , IKK $\beta$ , and IKK $\gamma$  expression in NDRG1/Cap43 and mock transfectants treated with MG-132 (10  $\mu$ mol/L) in the presence of 2% serum for indicated time. **D**, quantitative real-time PCR analysis of IKK $\beta$  mRNA levels in NDRG1/Cap43 and mock transfectants treated with MG-132 (10  $\mu$ mol/L) in the presence of 2% serum for the indicated time. Columns, mean of three independent experiments; bars, SE (top). Cells were treated with or without MG-132 (10  $\mu$ mol/L) for 8 h before lysis. Immunoprecipitation was done with anti-ubiquitin (Ub) antibody and immunoblotting was with anti-IKK $\beta$  antibody (bottom).



(Fig. 3D, top). We further examined whether IKK $\beta$  was ubiquitinated or not in the presence of MG-132. As shown in Fig. 3D (bottom), ubiquitination of IKK $\beta$  was shown in Cap#11 cells, suggesting that a proteasomal degradation plays a role in down-regulation of IKK $\beta$  in the NDRG1/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).

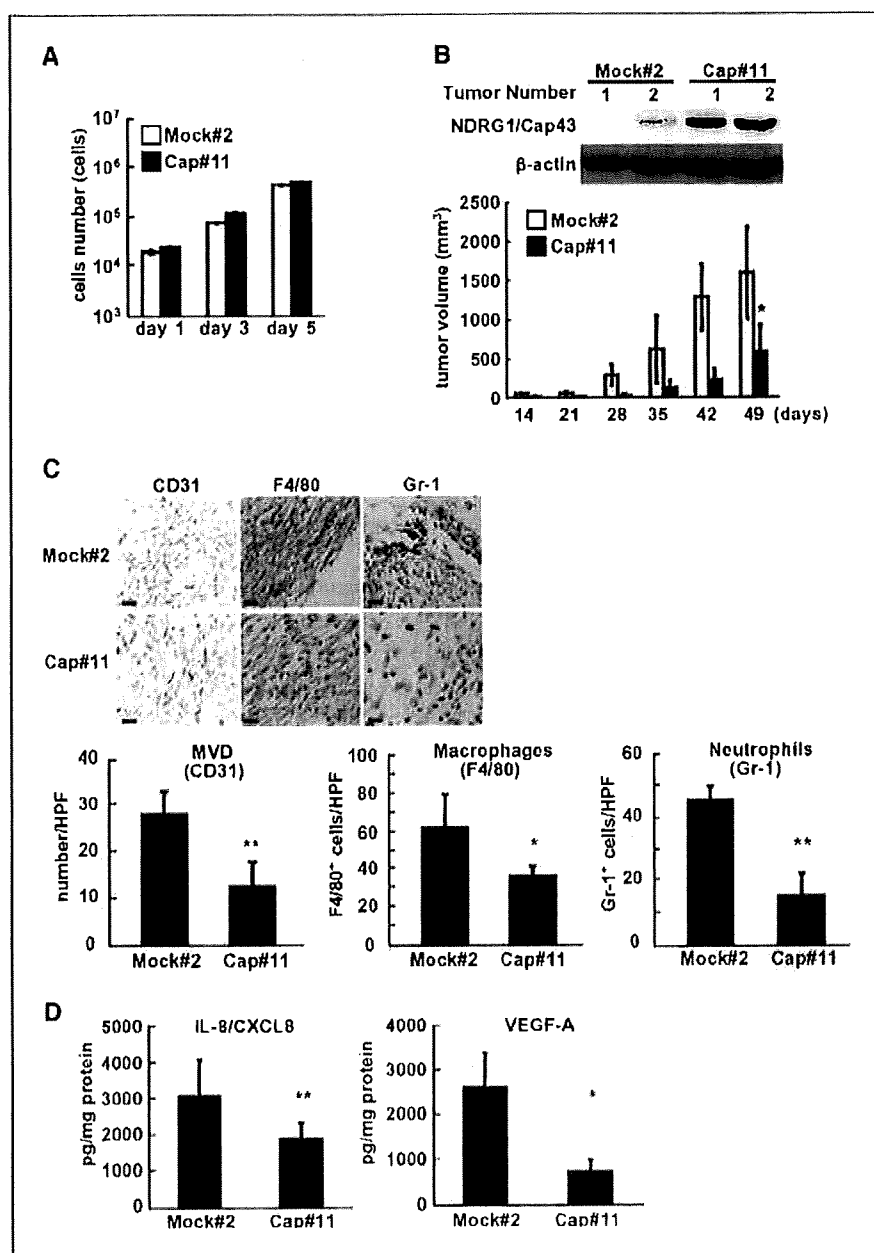
NDRG1/Cap43 was found to reduce the expression of chemokines and growth factors that function in chemotaxis of monocytes/macrophages and neutrophils (Fig. 1B; Supplementary Fig. S1). Mock#2 and Cap#11 tumor sections were further analyzed by immunohistochemistry for expression of microvessels (CD31), 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

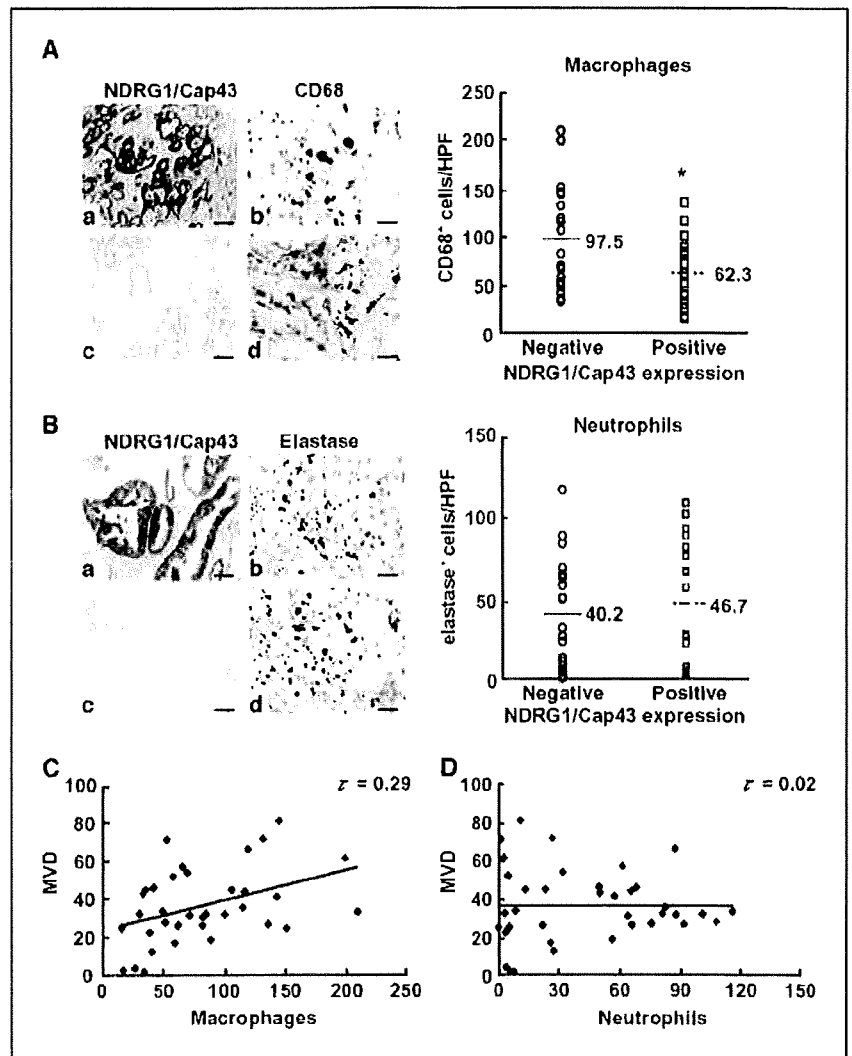
in patients with pancreatic ductal adenocarcinoma. High NDRG1/Cap43 expression was significantly correlated with invasion depth (Supplementary Table S3).

In the human tumor stroma, some cases showed a lower number of infiltrating CD68<sup>+</sup> macrophages/monocytes in NDRG1/Cap43-positive pancreatic cancer (Fig. 5A, a and b), whereas others showed a higher number of infiltrating CD68<sup>+</sup> 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



**Figure 4.** NDRG1/Cap43 represses MVD and the number of infiltrating macrophages or neutrophils in mouse subcutaneous tumors. **A**, comparison of cell proliferation in MIApaca-2 transfectant cells (Mock#2 and Cap#11). Cells were seeded on day 0 at  $1.0 \times 10^4$  per well and cultured in DMEM with 10% serum. Cell growth was measured on days 1, 3, and 5. Columns, mean of three independent experiments; bars, SE. **B**, Western blot analysis of NDRG1/Cap43 expression in tumors comprising Mock#2 or Cap#11 cells on day 49 (top). Columns, mean tumor volumes; bars, SE. Tumor volumes were determined every week after tumor implantation ( $n = 5$  or 6; bottom). **C**, Immunohistochemical analysis. Representative photographs of tumor sections stained with the anti-CD31, anti-F4/80, and anti-Gr-1 antibodies from Mock#2 and Cap#11 xenografts on day 49. Bar, 25  $\mu$ m (top). Mean MVD for tumor sections from Mock#2 and Cap#11 xenografts on day 49 was determined by counting the number of CD31<sup>+</sup> vessels in the tumors. Mean numbers of infiltrating macrophages and neutrophils in tumor sections from Mock#2 and Cap#11 xenografts on day 49 were determined by counting the intensity of F4/80- or Gr-1-positive cells in the tumor stroma. Columns, mean; bars, SE ( $n = 5$ ; bottom). HPF, high-power field. **D**, ELISA assay analysis of human IL-8/CXCL8 and VEGF-A protein levels in Mock#2 and Cap#11 tumors. Columns, mean; bars, SE ( $n = 4$ ). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus Mock#2 tumors.

**Figure 5.** NDRG1/Cap43 expression levels and numbers of infiltrating inflammatory cells in human pancreatic cancer. **A**, Immunohistochemical analysis of macrophages in tumor stroma using the anti-NDRG1/Cap43 antibody and anti-CD68 antibody. Representative photographs showing low infiltration of macrophages in NDRG1/Cap43-positive specimens (*a* and *b*). Representative photographs showing high infiltration of macrophages in NDRG1/Cap43-negative specimens (*c* and *d*). Bar, 50  $\mu$ m (*a* and *c*) and 25  $\mu$ m (*b* and *d*). Correlation between NDRG1/Cap43 expression levels and numbers of infiltrating macrophages. Mean number of infiltrating macrophages was 97.5 in NDRG1/Cap43-negative specimens ( $n = 19$ ) and 62.3 in NDRG1/Cap43-positive specimens ( $n = 18$ ; *right*). \*,  $P < 0.05$ . **B**, Immunohistochemical analysis of neutrophils in tumor stroma using an anti-NDRG1/Cap43 antibody and anti-elastase antibody. Representative photographs of neutrophils infiltration in NDRG1/Cap43-positive specimens (*a* and *b*) and NDRG1/Cap43-negative specimens (*c* and *d*). Correlation between NDRG1/Cap43 expression level and number of infiltrating neutrophils. Mean number of infiltrating neutrophils was 40.2 in NDRG1/Cap43-negative specimens and 46.7 in NDRG1/Cap43-positive specimens (*right*). **C** and **D**, correlation between MVD and number of infiltrating macrophages or neutrophils in patients with pancreatic ductal adenocarcinoma ( $n = 37$ ).  $r = 0.29$  and  $0.02$ , Kendall correlation coefficient.



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).

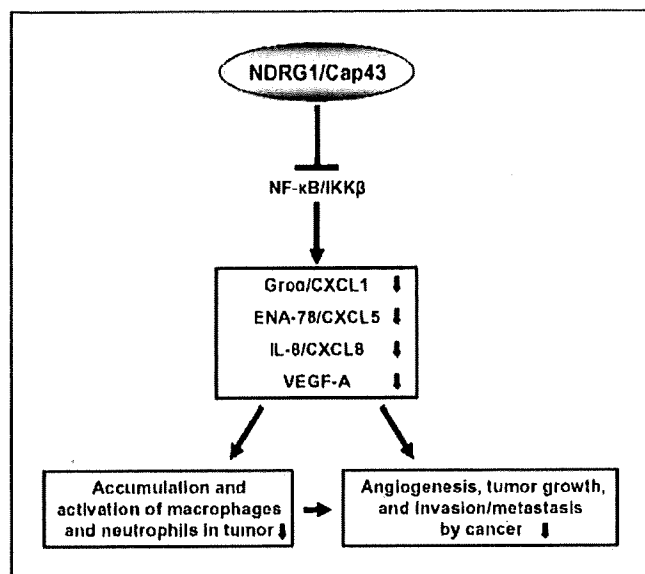
Our previous study showed that NDRG1/Cap43 expression levels were inversely correlated with MVD (5). Therefore, we further examined whether infiltration of macrophages/monocytes and neutrophils was associated with MVD in patients with NDRG1/Cap43-positive and NDRG1/Cap43-negative pancreatic cancer ( $n = 37$ ). 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 chemoattractants 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 chemoattractants 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). Gro $\alpha$ /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



**Figure 6.** NDRG1/Cap43 plays a critical role as an antiangiogenic regulator through modulation of the tumor microenvironment in pancreatic cancer. NDRG1/Cap43 attenuates activation of the NF- $\kappa$ B/IKK $\beta$  signaling, resulting in decreased expression of CXC chemokines (Gro $\alpha$ /CXCL1, ENA-78/CXCL5, and IL-8/CXCL8) and VEGF-A. NDRG1/Cap43 might thus remodel the tumor microenvironment by affecting the accumulation of inflammatory cells (macrophages and neutrophils), tumor angiogenesis, and tumor growth.

factors are directly involved in the NDRG1/Cap43-dependent suppression of inflammatory cell infiltration and angiogenesis.

Constitutive activation of NF- $\kappa$ B signaling pathway has been reported in many cancers, including pancreatic cancer (34). Fujioka and colleagues reported that pancreatic cancer cells expressing phosphorylation-defective I $\kappa$ B $\alpha$  showed decreased tumorigenicity in an orthotopic nude mouse model (35). In this mouse model, deletion of IKK $\beta$  in intestinal epithelial cells led to a decrease in tumor incidence without affecting tumor size (36). These studies suggested that the IKK $\beta$ -NF- $\kappa$ B signaling pathway plays an important role in tumor development.

In our present study, NDRG1/Cap43 reduced the expression of p-I $\kappa$ B $\alpha$  and its upstream regulator IKK $\beta$  in pancreatic cancer cells. However, we found no apparent phosphorylation of IKK $\alpha$  and IKK $\beta$  in NDRG1/Cap43 and mock transfectants under 2% serum condition (data not shown), suggesting that decreased expression of IKK $\beta$  is responsible for the loss of p-I $\kappa$ B $\alpha$  in NDRG1/Cap43 transfectants. The loss of p-I $\kappa$ B $\alpha$  results in reduction of both nuclear translocation of p65 and p50 and their binding to the NF- $\kappa$ B motif. NDRG1/Cap43-induced suppression of IKK $\beta$  was almost completely restored by a proteasome inhibitor. Introduction of an exogenous IKK $\beta$  gene was able to restore I $\kappa$ B $\alpha$  phosphorylation

and expression of Gro $\alpha$ /CXCL1, ENA-78/CXCL5, IL-8/CXCL8, and VEGF-A in NDRG1/Cap43 transfectants. In this study, we also found ubiquitination of IKK $\beta$  in NDRG1/Cap43 transfectant. A relevant study by May and colleagues showed that a ubiquitin-like domain of IKK $\beta$  is required for its functional activation (20). Taken together, the IKK $\beta$ -NF- $\kappa$ B 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 Cap#11 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 IKK $\beta$  and the NF- $\kappa$ B 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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## mTOR Signal and Hypoxia-Inducible Factor-1 $\alpha$ Regulate CD133 Expression in Cancer Cells

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### Abstract

The underlying mechanism regulating the expression of the cancer stem cell/tumor-initiating cell marker CD133/prominin-1 in cancer cells remains largely unclear, although knowledge of this mechanism would likely provide important biological information regarding cancer stem cells. Here, we found that the inhibition of mTOR signaling up-regulated CD133 expression at both the mRNA and protein levels in a CD133-overexpressing cancer cell line. This effect was canceled by a rapamycin-competitor, tacrolimus, and was not modified by conventional cytotoxic drugs. We hypothesized that hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), a downstream molecule in the mTOR signaling pathway, might regulate CD133 expression; we therefore investigated the relation between CD133 and HIF-1 $\alpha$ . Hypoxic conditions up-regulated HIF-1 $\alpha$  expression and inversely down-regulated CD133 expression at both the mRNA and protein levels. Similarly, the HIF-1 $\alpha$  activator deferoxamine mesylate dose-dependently down-regulated CD133 expression, consistent with the effects of hypoxic conditions. Finally, the correlations between CD133 and the expressions of HIF-1 $\alpha$  and HIF-1 $\beta$  were examined using clinical gastric cancer samples. A strong inverse correlation ( $r = -0.68$ ) was observed between CD133 and HIF-1 $\alpha$ , but not between CD133 and HIF-1 $\beta$ . In conclusion, these results indicate that HIF-1 $\alpha$  down-regulates CD133 expression and suggest that mTOR signaling is involved in the expression of CD133 in cancer cells. Our findings provide a novel insight into the regulatory mechanisms of CD133 expression via mTOR signaling and HIF-1 $\alpha$  in cancer cells and might lead to insights into the involvement of the mTOR signal and oxygen-sensitive intracellular pathways in the maintenance of stemness in cancer stem cells. [Cancer Res 2009;69(18):7160-4]

### Introduction

The CD133/prominin-1 protein is a five-transmembrane molecule expressed on the cell surface that is widely regarded as a stem cell marker. Growing evidence indicates that CD133 can be used as a cell marker for cancer stem cells or tumor-initiating cells in colon

cancer, prostate cancer, pancreatic cancer, hepatocellular carcinoma, neural tumors, and renal cancer (1). Strict regulatory mechanisms governing CD133 expression are thought to be deeply related to inherent cancer stemness; however, such mechanisms remain largely unclear, especially in cancer cells. In brain tumors, the Hedgehog (2), bone morphogenetic protein (3), and Notch (4) signaling pathways have been implicated in the control of CD133+ cancer stem cell function.

Some investigators have shown a relation between hypoxia and CD133 expression in brain tissue. The percentage of CD133-expressing cells was found to increase in a glioma cell line cultured under hypoxic conditions (5), and mouse fetal cortical precursors cultured under normoxic conditions exhibited a reduction in CD133(hi)CD24(lo) multipotent precursors and the failure of the remaining CD133(hi)CD24(lo) cells to generate glia (6). With the exception of these studies in brain tissue, however, data on the expression of CD133 and the involvement of hypoxia and other signaling pathways in cancer cells remains limited.

Several reports have indicated that mTOR is a positive regulator of hypoxia-inducible factor (HIF) expression and activity (7), and the inhibition of HIF-mediated gene expression is considered to be related to the antitumor activity of mTOR inhibitors in renal cell carcinoma (8). We found that mTOR signaling was involved in CD133 expression in gastric and colorectal cancer cells. Thus, we investigated the regulatory mechanism of CD133 in cancer cells.

### Materials and Methods

**Reagents.** 5-Fluorouracil, irinotecan (CPT-11), and rapamycin were purchased from Sigma-Aldrich. Gemcitabine was provided by Eli Lilly. Tacrolimus (LKT Laboratories), LY294002 and wortmannin (Cell Signaling Technology), and deferoxamine mesylate (DFC; Sigma-Aldrich) were purchased from the indicated companies.

**Cell cultures and hypoxic conditions.** All of the 28 cell lines used in this study were maintained in RPMI 1640 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), except for LoVo (F12; Nissui Pharmaceutical), WiDr, IM95, and HEK293 (DMEM; Nissui Pharmaceutical), and Huvec (Humedia; Kurabo). Hypoxic conditions (0.1% O<sub>2</sub>) were achieved using the AnaeroPouch-Anaero (Mitsubishi Gas Chemical) with monitoring using an oxygen indicator.

**Real-time reverse transcription-PCR.** The methods were previously described (9). The primers used for the real-time reverse transcription-PCR (RT-PCR) were as follows: CD133, forward 5'-AGT GGC ATC GTG CAA ACC TG-3' and reverse 5'-CTC CGA ATC CAT TCG ACG ATA GTA-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPD), forward 5'-GCA CCG TCA AGG CTG AGA AC-3' and reverse 5'-ATG GTG GTG AAG ACG CCA GT-3'. GAPD was used to normalize the expression levels in the subsequent quantitative analyses.

**Clinical samples.** The mRNA expression levels of CD133, HIF-1 $\alpha$ , and HIF-1 $\beta$  in gastric cancer specimens were obtained from previously published microarray data (9).

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

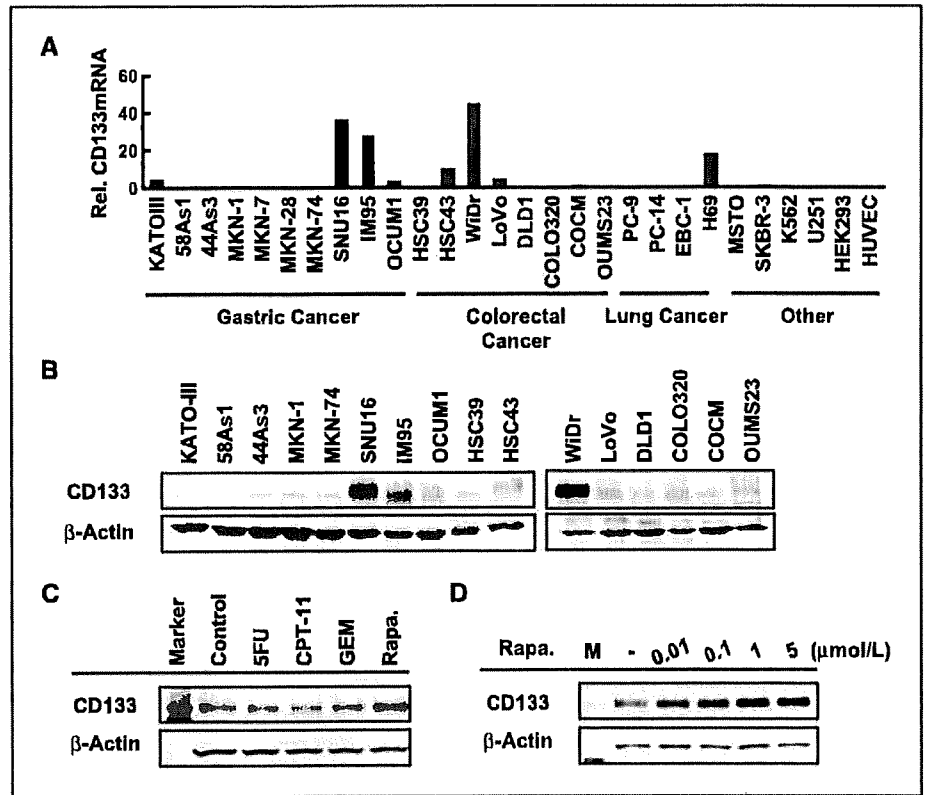
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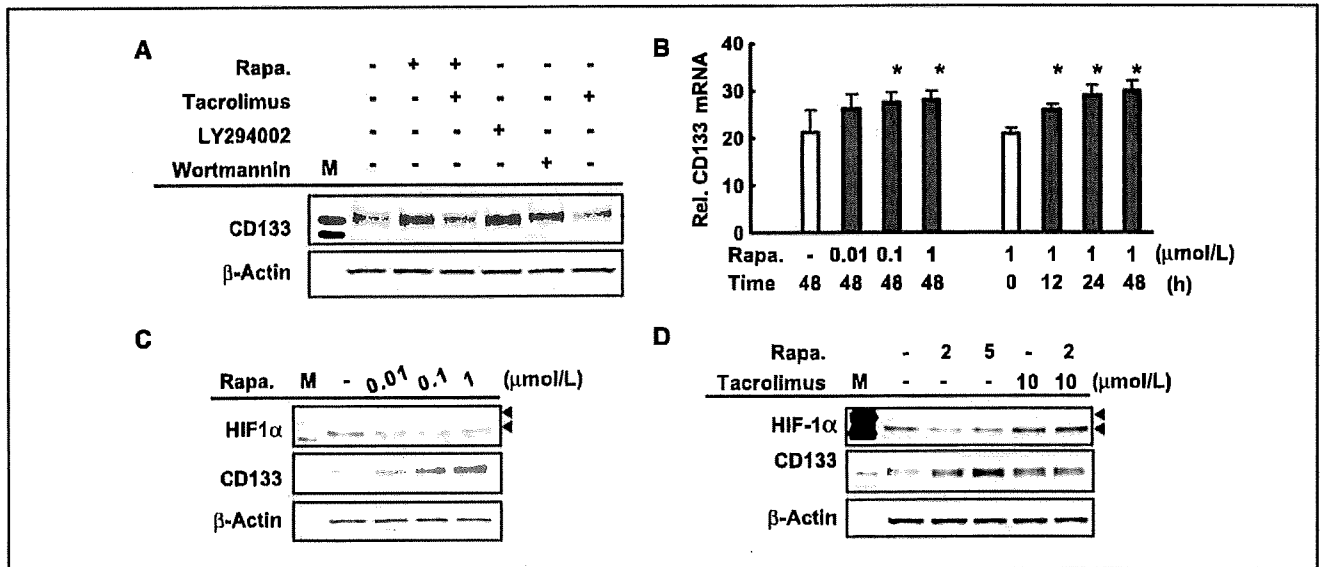
**Figure 1.** Rapamycin up-regulates CD133 expression. **A**, the mRNA expression levels of CD133 were examined using real-time RT-PCR in 26 cancer cell lines. **B**, the protein expressions of CD133 were determined using Western blotting in 16 gastric and colorectal cancer cell lines. **C**, Western blot of CD133 expression in WIDr cells exposed to cytotoxic drugs [1  $\mu$ mol/L of 5-fluorouracil (5-FU), CPT-11, and gemcitabine (GEM)] and rapamycin (1  $\mu$ mol/L) for 48 h. Note that only rapamycin up-regulates CD133 expression. **D**, WIDr cells were exposed to rapamycin at the indicated concentrations (0, 0.01, 0.1, 1, and 5  $\mu$ mol/L) for 48 h. Rapamycin dose-dependently up-regulated CD133 expression. *Rel. CD133 mRNA*, normalized mRNA expression levels (CD133/GAPD  $\times 10^4$ ); *Rapa.*, rapamycin.



**Immunoblotting.** A Western blot analysis was performed as described previously (10). The experiment was performed in triplicate. The following antibodies were used: monoclonal CD133 antibody (W6B3C1; Miltenyi Biotec), rabbit polyclonal HIF-1 $\alpha$  antibody (Novus Biologicals, Inc.),  $\beta$ -actin antibody, and HRP-conjugated secondary antibody (Cell Signaling Technology).

**Results**

**Inhibition of the mTOR signal up-regulates CD133 expression in CD133-overexpressing gastrointestinal cancer cells.** We examined the mRNA expression levels of CD133 in 26 cancer cell



**Figure 2.** Rapamycin down-regulates HIF-1 $\alpha$  expression and up-regulates CD133 expression at the transcriptional level. **A**, WIDr cells were exposed to rapamycin, the rapamycin-competitor tacrolimus, and the phosphoinositide-3-kinase inhibitors LY294002 and wortmannin for 48 h at concentrations of 10  $\mu$ mol/L. The inhibition of mTOR signaling up-regulated CD133 expression. **B**, rapamycin up-regulated the expression of CD133 mRNA in WIDr cells in a time-dependent and dose-dependent manner. *Columns*, mean determined using real-time RT-PCR; *bars*, SD. **C** and **D**, rapamycin exposure and HIF-1 $\alpha$  expression. WIDr cells were exposed to rapamycin with/without tacrolimus at the indicated concentration for 48 h. Rapamycin down-regulated HIF-1 $\alpha$  expression and inversely up-regulated CD133 expression; these effects were canceled by tacrolimus. *Rel. CD133 mRNA*, normalized mRNA expression levels (CD133/GAPD  $\times 10^4$ ); *Rapa.*, rapamycin.

lines using real-time RT-PCR. Several gastric, colorectal, and lung cancer cell lines such as SNU16, IM95, HSC43, WiDr, and H69, overexpressed CD133 (Fig. 1A). The increased expression of CD133 protein was also confirmed in these cell lines (Fig. 1B). The mTOR inhibitor rapamycin, but not cytotoxic drugs (5-fluorouracil, CPT-11, and gemcitabine), increased the expression of CD133 in a dose-dependent manner in CD133-overexpressing WiDr cells (Fig. 1C and D). These results indicate that mTOR signaling is involved in the expression of CD133 in cancer cells.

**Rapamycin down-regulated HIF-1 $\alpha$  expression and up-regulated CD133 expression at the transcriptional level.** To examine the signal transduction of rapamycin-induced CD133 expression, we used the rapamycin-competitor tacrolimus and the phosphoinositide-3-kinase inhibitors LY294002 and wortmannin. Tacrolimus (10  $\mu$ mol/L) completely canceled the up-regulation of CD133 induced by rapamycin. The inhibition of phosphoinositide-3-kinase by LY294002 (10  $\mu$ mol/L) and wortmannin (10  $\mu$ mol/L) also up-regulated CD133 expression (Fig. 2A). Rapamycin up-regulated CD133 expression at the transcriptional level in a dose-dependent and time-dependent manner (Fig. 2B).

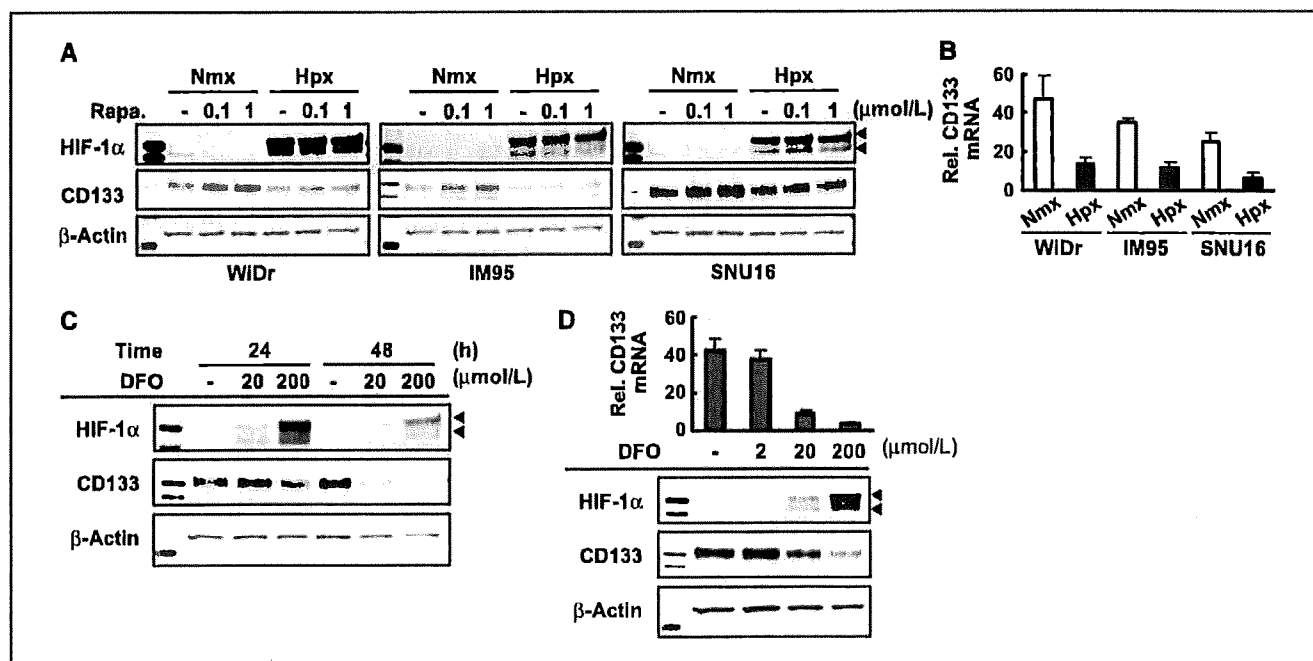
The inhibition of mTOR signaling is likely to lead to the down-regulation of the expression of certain molecules because the mTOR complex positively regulates the general translational machinery. Under the inhibition of mTOR signaling, HIF-1 $\alpha$ , among several downstream molecules of mTOR, can activate transcription by acting as a repressor of specific transcription factors such as the MYC-associated protein X homodimer (11). Therefore, we focused on the possible role of HIF-1 $\alpha$  in the regulation of CD133 expression. Rapamycin down-regulated HIF-1 $\alpha$  expression but up-regulated CD133 expression (Fig. 2C). Meanwhile, tacrolimus canceled the effect of rapamycin on the

expressions of HIF-1 $\alpha$  and CD133 (Fig. 2D). These results suggest that the down-regulation of HIF-1 $\alpha$  may mediate the up-regulation of CD133 expression in cancer cells. Up-regulation of CD133 expression by rapamycin was reproducibly observed in the CD133 high-expressing cell lines, but not in CD133 low-expressing cell lines (Supplemental Fig. S2).

**Induction of HIF-1 $\alpha$  down-regulates CD133 expression in cancer cells.** Hypoxia mediates the stabilization of HIF-1 $\alpha$  protein and enables its escape from rapid degradation, facilitating the up-regulation of HIF-1 $\alpha$  expression (12). Hypoxia strongly induced HIF-1 $\alpha$  expression, whereas CD133 expression was down-regulated in all three CD133-overexpressing cell lines (Fig. 3A). Rapamycin dose-dependently up-regulated CD133 expression under normoxic conditions, but no effect was seen under hypoxic conditions. We speculated that the effect of hypoxia on the induction of HIF-1 $\alpha$  is much higher than the effect of rapamycin on the down-regulation of HIF-1 $\alpha$ . The expression of CD133 mRNA was also strongly down-regulated under hypoxic conditions in all three cell lines (Fig. 3B) and in three additional cell lines (Supplemental Fig. S1).

In addition, DFO, a known HIF-1 $\alpha$  activator, induced HIF-1 $\alpha$  expression in a dose-dependent manner but down-regulated the expression of CD133 at both the mRNA and protein levels in WiDr cells (Fig. 3C and D), and in three additional cell lines (Supplemental Fig. S2). These results were consistent with those obtained under hypoxic conditions. Both hypoxia and DFO exposure markedly down-regulated CD133 expression, strongly suggesting that induction of HIF-1 $\alpha$  results in the down-regulation of CD133 expression.

**Inverse correlation between CD133 and HIF-1 $\alpha$  in clinical samples.** Finally, to address whether CD133 and HIF-1 $\alpha$  expression are inversely correlated in clinical samples of gastric cancer



**Figure 3.** Induction of HIF-1 $\alpha$  down-regulates CD133 expression in cancer cells. *A*, three gastrointestinal cancer cell lines were exposed to rapamycin under normoxic or hypoxic conditions for 24 h. Hypoxia induced HIF-1 $\alpha$  expression and inversely down-regulated CD133 expression. *B*, hypoxia strongly down-regulated CD133 expression at the mRNA level. Columns, mean determined using real-time RT-PCR; bars, SD. *C*, DFO, a known HIF-1 $\alpha$  activator, induced HIF-1 $\alpha$  expression and down-regulated CD133 expression in WiDr cells. *D*, DFO induced these effects at both the mRNA and protein levels. Note that both hypoxia and DFO exposure had similar effects on HIF-1 $\alpha$  induction and CD133 down-regulation. Rel. CD133 mRNA, normalized mRNA expression levels (CD133/GAPD  $\times 10^4$ ); Rapa., rapamycin.