# Infiltration of thymidine phosphorylase-positive macrophages is closely associated with tumor angiogenesis and survival in intestinal type gastric cancer

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**Abstract.** Thymidine phosphorylase (TP), an enzyme catalyzing the reversible phospholysis of thymidine, deoxyuridine and their analogs at their respective bases and 2-deoxyribose-1-phosphate, thus promoting angiogenesis, is often expressed in macrophages present in tumor stroma. In this study, we investigated whether infiltration of TP-positive macrophages as well as tumor-associated macrophages affected tumor angiogenesis. TP was expressed in human macrophage-like cells, but not in gastric cancer cells in culture. The expression level of TP, the number of infiltrating $CD68^+$ and $CD163^+$ macrophages, and microvessel density (MVD) in the tumor were further analyzed by immunohistochemistry in 111 patients with gastric cancer. Biostatistical analysis of digitized data obtained by image analysis showed that TP expression was significantly correlated with the number of infiltrating macrophages and MVD in intestinal type gastric cancer ($p<0.05$). The number of infiltrating macrophages was also correlated with MVD in both the intestinal and diffuse types ($p<0.05$). An increased number of $CD68^+$ macrophages was significantly associated with poor outcome in patients with intestinal type ($p<0.001$), but not diffuse type cancer. TP could be a specific marker enzyme that is expressed in tumor-infiltrating macrophages, being associated with tumor angiogenesis and poor prognosis in patients with intestinal-type gastric cancer.

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*Key words:* thymidine phosphorylase, tumor-associated macrophages, gastric cancer, tumor angiogenesis

## Introduction

Thymidine phosphorylase (TP) is an enzyme catalyzing the reversible phospholysis of thymidine, deoxyuridine and their analogs at their respective bases, and 2-deoxyribose-1-phosphate, thus playing a role in angiogenesis (1). The angiogenic activity of TP is due in part to production of reactive oxygen species by 2-deoxy-D-ribose, leading to up-regulation of angiogenic factors such as vascular endothelial growth factor and interleukin-8/CXCL8 through induction of oxidative stress and inflammatory signaling (2-4). TP is more highly expressed in differentiated than in undifferentiated gastric adenocarcinoma and patients whose tumors are TP-positive have a poorer prognosis than those with TP-negative tumors (5). Tumor expression of TP is significantly correlated with angiogenesis and invasion/metastasis in cancers of the colon and rectum, stomach, pancreas and bladder (6-9). We have previously observed a marked increase of TP expression in human macrophages in culture through transcriptional activation in response to inflammatory cytokines (10). We have also observed that in melanoma, the number of infiltrating macrophages increases depending upon invasion depth, in close association with angiogenesis (11), and that these macrophages are positive for TP expression (12). Shimaoka *et al* have further reported that TP expression in macrophages is correlated with the development of tumor microvessels, and also that TP plays an important role in tumor invasiveness and progression in differentiated gastric adenocarcinoma (8).

On the other hand, infiltration of macrophages in the tumor stroma often promotes the malignant progression of cancer through interaction with cancer cells (13,14). Among such macrophages, those activated by bacterial products and

interferon γ, known as inflammatory M1 type macrophages, have an IL-$2^{high}$, IN-$23^{high}$, IL-$10^{low}$ phenotype and produce NO, IL-1β, TNF-α and IL-6. By contrast, anti-inflammatory and activated macrophages, known as M2 type macrophages, have an IL-$12^{low}$, IL-$23^{low}$, IL-$10^{high}$ phenotype and produce pro-angiogenic factors and various receptors such as scavenger receptors and mannose receptors (15,16). These tumor-supporting macrophages are known as tumor-associated macrophages (TAMs), and support the processes of invasion, metastasis and angiogenesis by the production of inflammatory cytokines, chemokines, proteases, prostanoids, growth factors and angiogenic factors (13,14), suggesting that TAMs are rather more closely linked to the M2 type than to the M1 type. Clinical studies have demonstrated a close association between the abundance of TAMs and poor prognosis or tumor angiogenesis in colon cancer and glioma (17,18) as well as various other types of solid tumor including cancers of the breast, bladder and cervix (19). Various cytokines and growth factors are instrumental in the recruitment and accumulation of macrophages in the tumor stroma, and these macrophages then play a key role in the angiogenesis and malignant transformation (20). Depletion of these macrophages/TAMs by macrophage-targeting bisphosphonate encapsulated in liposomes markedly inhibits tumor growth, angiogenesis, and bone metastasis (21-23), thus corroborating the involvement of macrophages in these cancer-related activities (14). Tumor-supporting macrophages such as TAMs can often be differentially characterized from tumor-suppressive macrophages. Tumor-supportive macrophages are active in extracellular matrix remodeling, tissue repair and angiogenesis, whereas tumor-suppressive macrophages are active in antimicrobial and antitumor activities through immunostimulatory functions (24-26). In patients with gliomas we have previously reported that the number of macrophages in stage IV glioblastoma is higher than that in grade II or III glioma, and is also closely correlated with microvessel density in the tumors (27).

Concerning the role of macrophages/TAMs in gastric cancer, infiltrating macrophages have been associated with a good prognosis, suggesting a beneficial effect of TAM aggregation in advanced gastric cancer (28). However, it has not been determined whether the effect of TAMs is dependent on the histological type of gastric cancer. Migita *et al* have reported that the number of macrophages along the invasive margin is significantly greater in patients without, than in those with, liver metastasis (29). Although no significant correlation has been observed between the number of macrophages and the depth of cancer invasion or lymph node metastasis, a significant association between macrophage number and the survival of patients with intestinal type gastric cancer has been reported (29). With regard to the classification of gastric cancer (30), it has been reported that the intestinal type tends to metastasize through blood vessels, whereas the diffuse type tends to cause peritoneal dissemination and/or lymph node metastasis (31). However, it still remains unknown whether tumor-supporting macrophages such as TAMs play a role in the malignant progression of gastric cancer in relation to histological type.

In the present study, we screened the expression of TP in human cancer cell lines and macrophage-like cell lines by Western blot analysis. Our aim was to determine whether TP could be a specific biomarker of infiltrating macrophages in gastric cancer, and also whether such macrophages could affect angiogenesis, lymph node metastasis and peritoneal dissemination, as well as patient survival. We also discuss the possible relationship of TP-expressing macrophages with tumor angiogenesis and other malignant characteristics.

## Materials and methods

*Cell culture.* The human gastric cancer cell lines MKN-28, MKN-45, MKN-74, AZ-521, NUGC-3 and KATO-III were cultured with RPMI supplemented with 10% fetal bovine serum (FBS) and incubated in humidified atmosphere of 5% $CO_2$ at 37˚C. The macrophage cell lines THP-1 and U937 were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI supplemented with 10% FBS and incubated in humidified atmosphere of 5% $CO_2$ at 37˚C. We also used KB/TP expressing high amount of TP (3,4).

*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 $Na_3VO_4$. Cell lysates were subjected to SDS-PAGE and blotted onto Immobilon membranes (Millipore Corp., Bedford, MA) as described previously. 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). The intensity of the luminescence was quantified using a CCD camera combined with an image analysis system (LAS-1000; Fuji Film, Japan).

*Patients and tumor samples.* We examined 111 patients with advanced gastric cancer whose tumors had been surgically removed in the Department of Surgery of Kurume University, between 2001 and 2004. The age of the gastric cancer patients ranged from 33 to 86 years (median, 69), 77 were men and 34 were women. Histological types were performed according to the criteria of Lauren classification (21), and tumor stage was performed according to the TNM classification. Patient characteristics are summarized in Table I. Cancer stages were 18.0% Stage I (IA+IB), 14.4% Stage II, 22.5% Stage III (IIIA+IIIB) and 45.0% Stage IV. At the time of surgery, 73 (65.8%), 17 (15.3%) and 26 (23.4%) patients had lymph node metastasis, liver metastasis and peritoneal dissemination, respectively. No patients had been administered drugs before surgery, and the standard chemotherapy was performed after surgery: Stage II or III patients were administered by TS-1, and Stage IV patients were administered by a combination of TS-1 and cisplatin.

*Immunohistochemistry (IHC) analysis.* Paraffin-embedded tissue samples were cut at 4 μm and examined on a coated slide glass and labeled with the following antibodies using the BenchMark XT (Ventana Automated Systems, Inc., Tucson, AZ, USA) and ChemMate Envision methods

Table I. Clinicopathological characteristics in gastric cancer patients.

| | Histological type | | |
|---|---|---|---|
| | Total (%) (n=111) | Intestinal (n=57) | Diffuse (n=54) |
| Age | | | |
| Median (min-max) | 69 (33-86) | 67.5 (33-86) | 69 (39-85) |
| Gender | | | |
| Male | 77 (69.4) | 47 | 30 |
| Female | 34 (30.6) | 10 | 24 |
| Stage | | | |
| I | 20 (18.0) | 11 | 9 |
| II | 16 (14.4) | 9 | 7 |
| III | 25 (22.5) | 13 | 12 |
| IV | 50 (45.0) | 24 | 26 |
| Lymph node metastasis | | | |
| Present | 73 (65.8) | 37 | 36 |
| Absent | 38 (34.2) | 20 | 18 |
| Liver metastasis | | | |
| Present | 17 (15.3) | 14 | 3 |
| Absent | 94 (84.7) | 43 | 51 |
| Peritoneal dissemination | | | |
| Present | 26 (23.4) | 7 | 19 |
| Absent | 85 (76.6) | 50 | 35 |

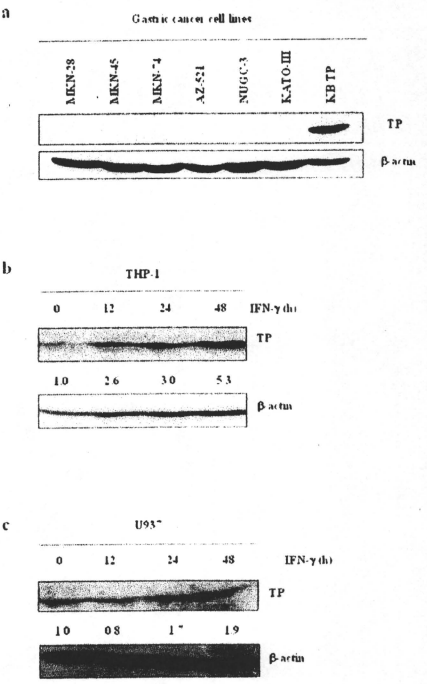


Figure 1. Expression levels of TP in 6 human gastric cancer cell lines (a) and 2 human macrophage-like cell lines (b, c) in culture. Macrophages were treated with 50 U/ml of IFNγ. Western blot analysis of TP by specific antibody against TP was performed. Protein (100 μg) of total cell lysates was loaded. Quantitative analysis for TP expression is presented when TP level in macrophages treated with INFγ for 0 h is normalized as 1.0.

(DakoCytomation, Glostrup, Denmark), TP (x2000, antibodies supplied by Taiho Pharmaceutical Co., Ltd., Tokyo Japan), CD68 (x1200, KP-1, DakoCytomation), CD163 (x100, Novocastra, Newcastle, UK) and CD34 (x200, Novocastra). The ChemMate Envision method was used for TP, CD68 and CD34. Endogenous peroxidase activity was inhibited by incubating the slides in 3% $H_2O_2$ for 5 min. CD68 and CD34 antigen retrieval was performed by treating with proteinase K for 5 min. Each slide was incubated for 30 min with the antibody at room temperature. For staining detection, the ChemMate Envision method was used with DAB as chromogen. For CD163, BenchMark XT was used. This automated system used the streptavidin biotin complex method with DAB as chromogen (Ventana iView DAB Detection kit).

*IHC expression area analysis for TP, infiltration macrophages and MVD assessment.* We extracted the digital data of the expression using the following image analysis systems. TP, CD68, CD163 and CD34-stained specimens were examined to identify the areas with the high density. Images of the expression area were selected for clarity from 6 fields at x200 for each IHC specimen, using a CCD digital camera (Nikon, DXM1200). Expression analysis was performed to measure the expression area of TP, number of macrophages and MVD in all cases, using 'Win ROOF' (version 5.7; Mitani Corp., Osaka, Japan) computer software. The digitized data of the expression area were measured and averaged. The expression of TP often showed not only cancer cells, but also cancer stroma cells, such as macrophages, fibroblasts or vessels. The expression of TP was investigated, including both cancer cells and stroma cells.

*Determination of co-expression of TP and CD68.* We extracted the digital data of the expression levels using the following image analysis systems in all IHC specimens. Co-expression of TP and CD68 was revealed by the Nuance System (Cambridge Research and Instrumentation Inc., Woburn, MA, USA) (32). Double-stained slides for TP and CD68 antibodies were imaged at x200 through a liquid crystal tunable filter. TP and CD68 were labeled with a very intensely purple (VIP) substrate kit (Vector Laboratories, Burlingame, CA, USA) and

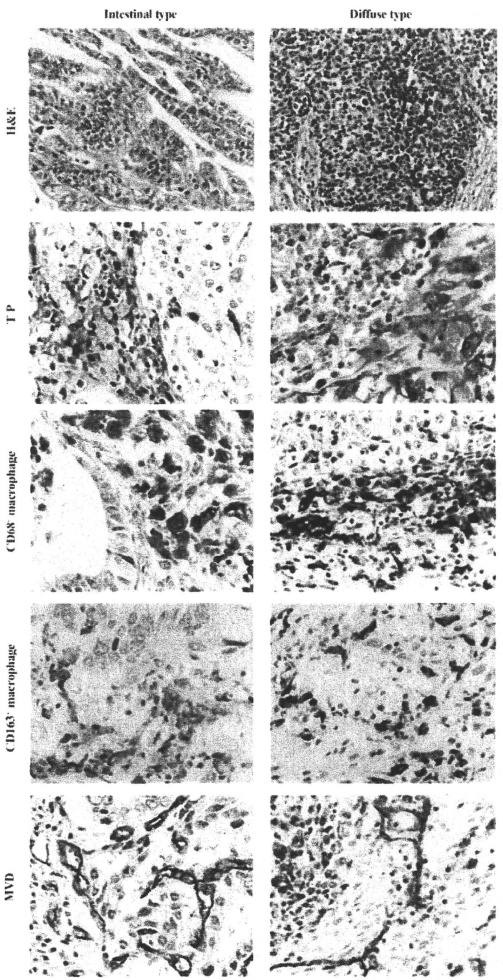


Figure 2. Representative images of immunohistochemical analysis of gastric cancer patients with intestinal and diffuse type (x200). Expression of TP, CD68, CD163 and CD34 is observed in cancer stroma in both histological types.

DAB as chromogen, respectively. Counterstaining was with hematoxylin. Multispectral image data were acquired from 420 to 720 nm at x200 in 3 fields from double-stained slides of 10 patients selected at random. Spectral unmixing was accomplished using Nuance software v2.8 and pure spectral libraries of individual chromogens. Expression areas of TP, CD68 and TP-CD68 co-expression were evaluated using Nuance software.

*Statistical analysis.* Associations among TP, CD68, CD163 and CD34 were evaluated using Spearman's rank correlation coefficients. The overall survival was defined as days from surgery until death due to any cause. Log-rank tests and Kaplan-Meier methods were applied to examine the effect of TP (CD68, CD163 and CD34) on overall survival classifying patients into negative and positive according to their TP (CD68, CD163 and CD34) were less than median or equal

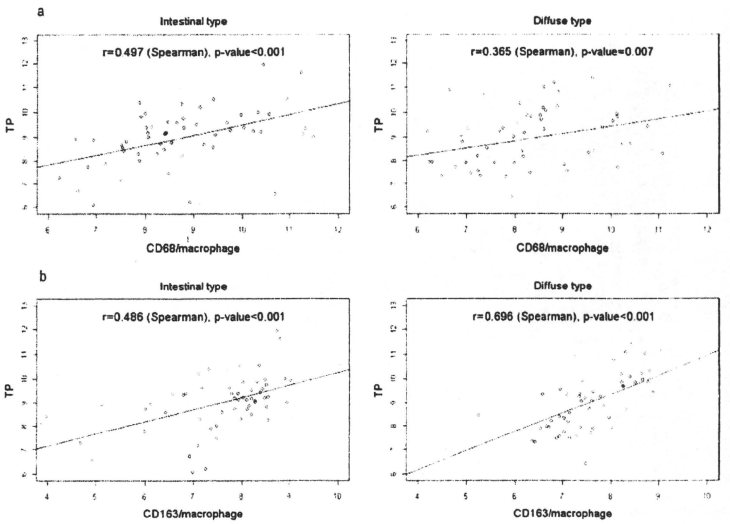


Figure 3. Correlation with TP and infiltrating macrophages (CD68 and CD163) in intestinal type (a) and diffused type (b) of gastric cancer.

to or more than median respectively. In addition, the Cox proportional hazards models were applied to examine the effect of TP (CD68, CD163 and CD34) on overall survival adjusting for possible confounding factors. The effects of TP (CD68, CD163 and CD34) on lymph node metastasis, liver metastasis and peritoneal dissemination were examined by logistic regression. In logistic regression analysis and Cox regression analysis, TP, CD68, CD163 and CD34 were not categorized into two categories of negative and positive and were log-transformed since they were highly skewed. When adjusting for possible confounding factors, Stage was not adjusted for since it might be an intermediate variable between TP (CD68, CD163 and CD34) and overall survival (33). Statistical analysis was performed by SAS version 9.1 (SAS Institute Inc., Cary, NC), StatXact (Cytel Inc., Cambridge, MA) and R version 2.8.1.

## Results

*Expression of TP in macrophage-like cell lines.* TP is known to be expressed in both cancer cells and macrophages in the tumor stroma of gastric cancer (4). We first examined the expression of TP in human gastric cancer cell lines and macrophage-like cells in culture. Western blot analysis demonstrated no apparent expression of TP in any of the gastric cancer cell lines (Fig. 1a). By contrast, macrophage-like THP-1 and U937 cells expressed TP. Expression of TP was further stimulated by the inflammatory cytokine interferon γ (Fig. 1b and c). TP was expressed rather specifically in macrophages relative to its expression in gastric cancer cells, consistent with the results of our previous study (10).

*Expression of TP in infiltrating macrophages in relation to microvessel density in intestinal and diffuse type gastric cancer.* To determine whether TP could be a specific marker enzyme of macrophages, we examined the expression of TP in tumor stromal macrophages as well as cancer cells in clinical specimens of gastric cancer using IHC. Fig. 2 shows representative IHC images for TP, $CD68^+$ and $CD163^+$ macrophages and MVD in gastric cancers. TP was expressed mainly in cancer stromal cells. The numbers of infiltrating macrophages and MVD were found to be higher in cancerous than in non-cancerous regions. Box plots based on quantitative determination of the expression levels of various biomarkers by IHC analysis showed that the expression levels of TP, $CD68^+$ macrophages, $CD163^+$ macrophages and MVD were all similar between intestinal-type and diffuse-type gastric cancers (data not shown).

*Close association of TP with infiltrating macrophages and TAMs in gastric cancer.* CD68 is a known marker by which all monocytes/macrophages can be recognized. By contrast, CD163 is monocyte/macrophage-specific membrane protein that is closely associated with the anti-inflammatory M2 macrophage phenotype, and is a useful marker for distinguishing M2 macrophages that are linked to TAMs from pro-inflammatory M1 macrophages (18). Fig. 3 shows cross-plots between TP and infiltrating $CD68^+$ and $CD163^+$ macrophages with Spearman's rank correlation coefficents in patients with both intestinal and diffuse type gastric cancer. There was a positive correlation between TP and CD68 in both intestinal type (Spearman's rank correlation $r=0.497$, $p<0.001$) and diffuse type ($r=0.365$, $p=0.007$) cancer. Furthermore, there

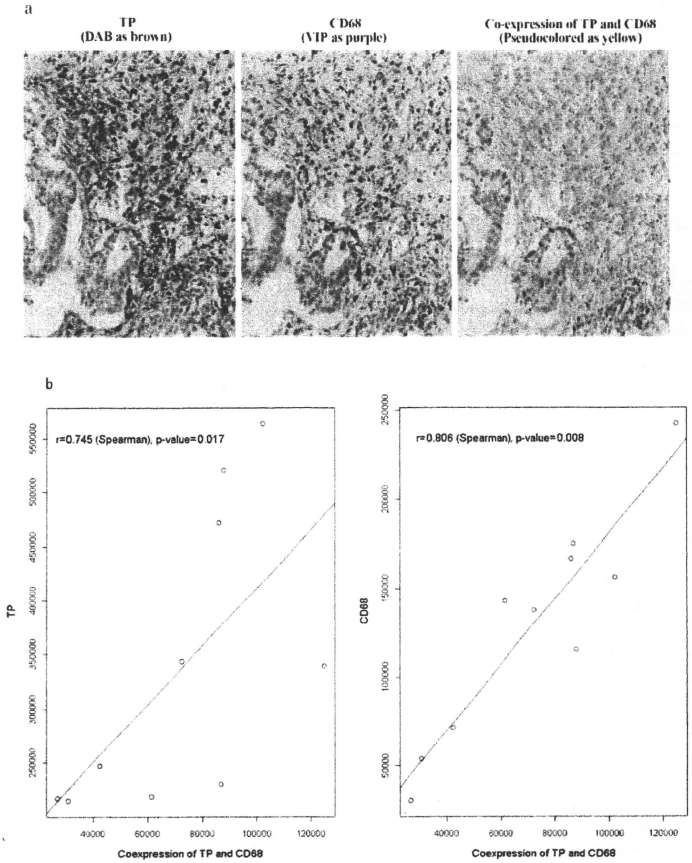


Figure 4. The quantitative analysis of close association of TP expression and $CD68^+$ macrophage in IHC images of gastric cancer. $CD68^+$ macrophage was labeled with VIP (purple) and TP was labeled with DAB (brown), and the co-expression of CD68 and TP was pseudocolored as yellow (a). Panel b is a cross plot of the average co-expression area of CD68 and TP (y-axis) over the average expression area of CD68 (x-axis) was presented for 10 randomly selected patients. The average expression area was calculated from three points for each specimen (b).

was a positive correlation between TP and CD163 in both intestinal-type ($r=0.486$, $p<0.001$) and diffuse type ($r=0.696$, $p<0.001$) cancer. Thus in gastric cancers there was a positive correlation between TP and infiltrating macrophages/TAMs.

Fig. 4a shows the expression of TP in macrophages in representative IHC images. TP was found to be expressed in macrophages that infiltrated gastric cancers. TP was labeled brown with DAB, and $CD68^+$ macrophages were labeled purple with VIP. Co-expression of TP and CD68 was pseudocolored in yellow, indicating that most macrophages expressed TP (TP-positive macrophages). Quantitative analysis of specimens from 10 patients indicated that the area of co-expression of CD68 and TP showed a statistically significant positive correlation with the area of expression of DAB-labeled TP ($r=0.745$, $p=0.017$) and VIP-labeled CD68 ($r=0.806$, $p=0.008$) (Fig. 4b).

*TP and infiltrating macrophages affect tumor angiogenesis in gastric cancers.* We then investigated the relationship between TP, infiltrating macrophages and MVD in gastric cancers. Fig. 5 shows cross-plots among TP, infiltrating macrophages and MVD with Spearman's rank correlation co-

Table II. Correlation of TP, MVD and infiltrating macrophage number with lymph node metastasis, liver metastasis and peritoneal dissemination in intestinal type.

| | Intestinal type of gastric cancer | | |
|---|---|---|---|
| | P-value | OR | 95% CI |
| Lymph node metastasis | | | |
| TP | 0.040 | 1.898 | (1.029, 3.502) |
| CD68+ macrophage | 0.093 | 2.091 | (0.885, 4.943) |
| CD163+ macrophage | 0.003 | 4.307 | (1.657, 11.197) |
| MVD | 0.431 | 1.531 | (0.530, 4.417) |
| Liver metastasis | | | |
| TP | 0.326 | 1.426 | (0.702, 2.894) |
| CD68+ macrophage | 0.103 | 2.166 | (0.856, 5.481) |
| CD163+ macrophage | 0.856 | 0.932 | (0.436, 1.991) |
| MVD | 0.059 | 3.029 | (0.959, 9.564) |
| Peritoneal dissemination | | | |
| TP | 0.612 | 0.818 | (0.377, 1.777) |
| CD68+ macrophage | 0.149 | 2.299 | (0.743, 7.115) |
| CD163+ macrophage | 0.749 | 1.166 | (0.456, 2.979) |
| MVD | 0.660 | 0.714 | (0.158, 3.216) |

Odds ratio (OR) and its 95% CI are those of patients with 75% quantile of TP, macrophage number and MVD to those with 25% quantile. OR is calculated by fitting logistic regression model with TP, macrophage number and MVD, age and gender, where TP, macrophage and MVD were log-transformed.

efficients in intestinal and diffuse type gastric cancers. There was a significant positive correlation between TP and MVD in intestinal type (r=0.338, p=0.010) gastric cancer, but not diffuse type (r=0.209, p=0.129) gastric cancer (Fig. 5a). There was a positive correlation between CD68+ macrophages and MVD in both intestinal (r=0.750, p<0.001) and diffuse type (r=0.553, p<0.001) gastric cancer (Fig. 5b). Similarly, there was a positive correlation between CD163+ macrophages and MVD in both intestinal (r=0.307, p=0.021) and diffuse type (r=0.272, p=0.046) cancer (Fig. 5c). Thus, infiltrating CD68+ and CD163+ macrophages are positively associated with MVD in both intestinal and diffuse type gastric cancer.

*TP and infiltrating macrophages are associated with metastasis and/or survival of cancer patients.* We examined whether lymph node metastasis, liver metastasis and peritoneal dissemination were associated with TP, infiltrating macrophages and MVD (Table II). In intestinal type cancer, TP (p=0.040) and CD163+ macrophages (p=0.003) were significantly associated with the occurrence of lymph node metastasis. By contrast, TP, infiltrating CD68+ and CD163+ macrophages and MVD were unassociated with metastases in diffuse type cancer (data not shown).

We also examined whether the expression of TP, infiltrating macrophages and MVD was associated with the overall survival of gastric cancer patients. P-values of the log-rank tests for TP, infiltrating macrophages and MVD are summarized in Table III. In intestinal type cancer, the association of CD68+ macrophages was statistically significant (p<0.001), and that of MVD was nearly significantly (p=0.060). Table III also gives the p-values and hazard ratio estimates with Cox regression adjustment for age and gender as possible confounding factors. The number of CD68+ macrophages had a significant effect on overall survival (p=0.010), and the p-value for MVD was close to statistical significance (p=0.060) even after adjusting for possible confounding factors.

Kaplan-Meier estimates of overall survival for patients with intestinal type and diffuse type cancer are presented in Fig. 6 in relation to CD68+ macrophage status. Patients with the intestinal type and an increased number of CD68+ macrophages showed poor overall survival (Fig. 6a), whereas for patients with the diffuse type there was no apparent association of CD68+ macrophages with overall survival (Fig. 6b).

Table III. Effects of TP, infiltrating macrophage number and MVD on overall survival by log-rank test and Cox regression in gastric cancers.

| | Intestinal type | | | | Diffuse type | | | |
|---|---|---|---|---|---|---|---|---|
| | Log-rank test | Cox regression | | | Log-rank test | Cox regression | | |
| | P-value | P-value | HR | 95% CI | P-value | P-value | HR | 95% CI |
| TP | 0.476 | 0.845 | 0.965 | (0.678, 1.375) | 0.380 | 0.817 | 0.935 | (0.531, 1.647) |
| CD68+ macrophage | <0.001 | 0.010 | 2.045 | (1.183, 3.535) | 0.487 | 0.587 | 0.847 | (0.467, 1.539) |
| CD163+ macrophage | 10.089 | 0.242 | 1.300 | (0.838, 2.017) | 0.918 | 0.752 | 0.926 | (0.574, 1.493) |
| MVD | 0.060 | 0.060 | 1.938 | (0.973, 3.860) | 0.089 | 0.249 | 0.727 | (0.423, 1.250) |

Hazard ratio (HR) and its 95% CI are those of patients with 75% quantile of TP, macrophage number and MVD to those with 25% quantile. HR is calculated by fitting Cox regression model with TP, macrophage and MVD, age and gender where TP, macrophage and MVD were log-transformed.

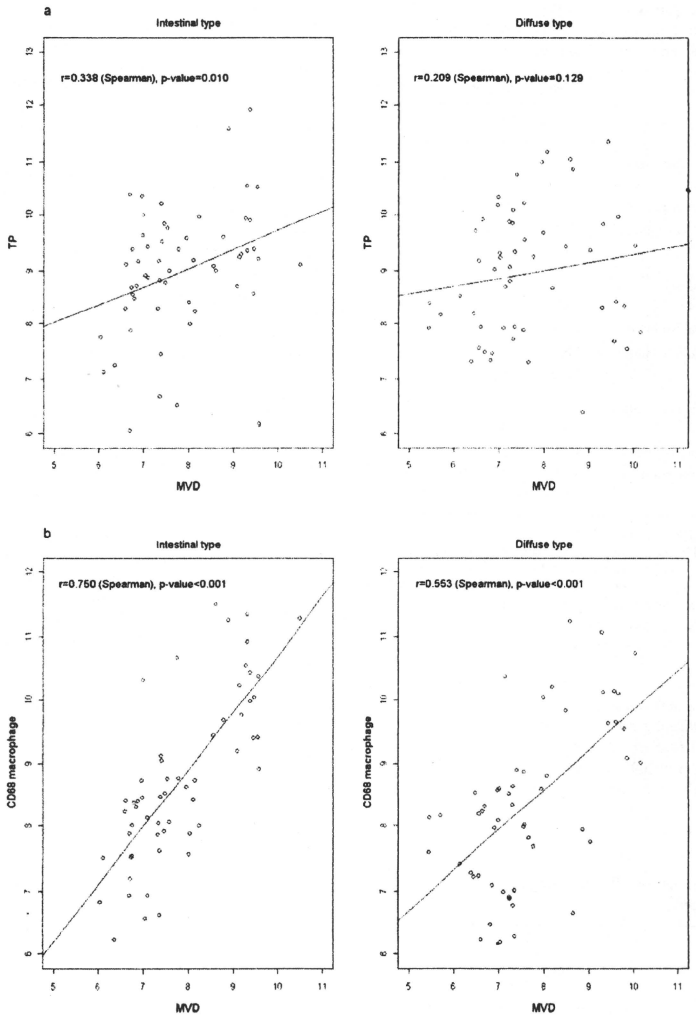


Figure 5. a and b. Correlation with MVD and TP (a) or CD68+ macrophage (b).

## Discussion

In our present study, we demonstrated that expression of TP in macrophages may affect tumor angiogenesis and prognosis in patients with intestinal type gastric cancer. i) Expression of TP was observed in macrophages infiltrating both intestinal and diffuse type gastric cancer; ii) Expression of TP and the number of infiltrating macrophages were significantly associated with tumor angiogenesis in intestinal-type gastric cancer; iii) The number of infiltrating macrophages was significantly associated with overall survival in patients with intestinal-type gastric cancer.

Infiltrating macrophages were closely correlated with TP expression and tumor angiogenesis. Our present findings

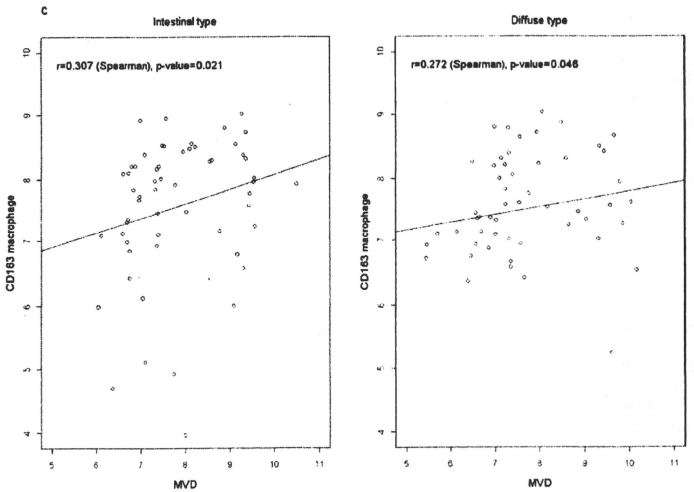


Figure 5. Correlation with MVD and TP (a) or CD68$^+$ macrophage (b) or CD163$^+$ macrophage (c) in intestinal type (left) and diffuse type (right) of gastric cancer.

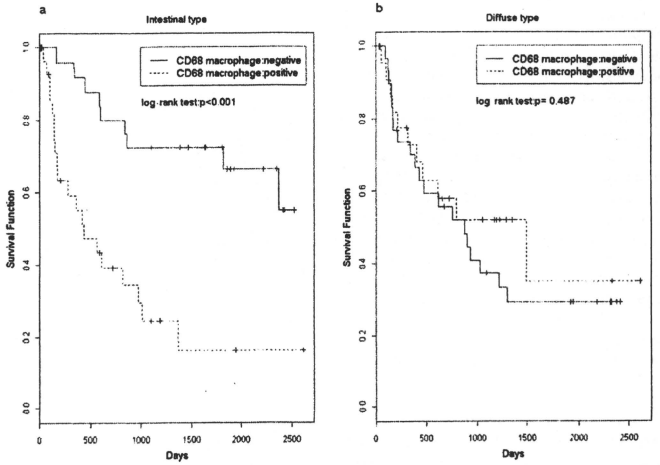


Figure 6. Kaplan-Meier estimators for overall survival in intestinal type (a) and diffuse type (b) of gastric cancer according to higher (---) and lower (—) infiltration of macrophage status with p-value by the log-rank test. In patients with higher number of infiltrating macrophages, increased number of macrophages have statistically significantly poor prognosis for overall survival.

suggest that the expression of TP in infiltrating macrophages and TAMs affects tumor angiogenesis in intestinal-type gastric cancer. The presence of extensive macrophage/TAM infiltration in tumors has often been shown to correlate with poor prognosis in cancers of the breast, cervix and bladder, and also in melanoma (19). However, the possible role of

infiltrating macrophages/TAMs in the progression of gastric cancer remains unknown. Concerning possible predictive markers of macrophages or TAMs in gastric cancer, Migita *et al* have reported that the number of CD68$^+$ macrophages tends to be associated with better patient survival, but not to a significant degree (29). Furthermore, Ohno *et al* have reported that the degree of CD68$^+$ macrophage infiltration in cancer cell nests is a significant predictor of better prognosis in patients with gastric cancer (28). At present, it remains unclear why infiltration of CD68$^+$ macrophages tends to be a marker of favorable prognosis only in gastric cancer, whereas it is a marker of poor prognosis in other human malignancies (see Introduction) (19). In the present study, however, the number of CD68$^+$ macrophages was found to be significantly correlated with poor prognosis in patients with intestinal type cancer, but not in patients with diffuse type cancer, suggesting that infiltration of tumor-associated macrophages could affect malignant progression and prognosis according to the histological type of gastric cancer. Furthermore, tumor angiogenesis was also closely associated with infiltration of CD68$^+$ or CD163$^+$ macrophages (Fig. 5). Based on these findings, we consider that infiltration of macrophages/TAMs affects the prognosis of intestinal type gastric cancer. However, a more detailed study will be necessary to clarify how tumor-associated macrophages differentially modulate prognosis or malignant progression in intestinal-type and diffuse-type gastric cancer.

Akiyama and colleagues have previously reported rather specific expression of TP in macrophages relative to cancer cells in differentiated gastric adenocarcinoma (4,8), and patients with TP-positive carcinomas have a poorer prognosis than those with TP-negative differentiated adenocarcinomas. Our present study also demonstrated that tumor-infiltrating macrophages are a potent prognostic marker in intestinal-type, but not diffuse-type, gastric cancer. Inflammatory stimuli markedly enhance the expression of TP in macrophages through transcriptional activation (Fig. 1) (10). Expression of TP results in enhanced production of reactive oxygen species by 2-deoxy-D-ribose, a downstream mediator of TP, indicating that TP induces oxygen stress (2,4,34). TP-positive macrophages are thus expected to be highly active, not only in the production of reactive oxygen, but also various angiogenesis factors, growth factors, chemokines and proteases, indicating that such macrophages are highly angiogenic. In fact, through cross-talk with cancer cells, macrophages are induced to undergo transformation into tumor-supporting macrophages that actively stimulate angiogenesis and cancer cell invasion (13,14,20). Thus, the expression of TP in macro-phages might play a key role in angiogenesis, tumor growth and anti-apoptosis in malignant cancers in the stomach as well as those of the colon and other organs, possibly through the enhanced production of angiogenesis factors by macrophages (2,4). In both intestinal and diffuse type gastric cancer, the number of macrophages was significantly correlated with tumor angiogenesis, and TP expression was also positively correlated with the presence of both CD68$^+$ and CD163$^+$ macrophages. By contrast, TP expression was significantly associated with angiogenesis only in intestinal type gastric cancer, and not in the diffuse type. A further study will be needed to clarify why TP expression thus differentially affects angiogenesis in gastric cancer depending on its histological type.

In conclusion, our present study demonstrates a close association of TP expression in tumor-infiltrating macrophages with angiogenesis in gastric cancer, lending support to the idea that TP-positive macrophages promote angiogenesis and metastasis in this cancer. TP expression in macrophages could be a useful and novel biomarker for diagnosis of gastric cancer and the development of new therapeutic strategies.

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# Identification of sites subjected to serine/threonine phosphorylation by SGK1 affecting N-myc downstream-regulated gene 1 (NDRG1)/Cap43-dependent suppression of angiogenic CXC chemokine expression in human pancreatic cancer cells

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ABSTRACT

We have recently reported that N-myc downstream-regulated gene 1 (NDRG1)/$Ca^{2+}$-associated protein with a molecular mass of 43 kDa (Cap43) suppresses angiogenesis and tumor growth of pancreatic cancer through marked decreases in both the expression of CXC chemokines and phosphorylation of a NF-κB signaling molecule, inhibitor of κB kinase (IκBα). NDRG1/Cap43 is phosphorylated at serine/threonine sites in its C-terminal domain by serum- and glucocorticoid-regulated kinase 1 (SGK1). In this study, we attempted to clarify the domain or site of NDRG1/Cap43 responsible for its suppression of CXC chemokine expression in pancreatic cancer cells. Expression of the deletion constructs CapΔ2 [deletion of amino acids (AA) 130–142] and CapΔ4 [deletion of AA 180–294] as well as the wild-type full sequence of NDRG1/Cap43 (F-Cap), suppressed the production of CXC chemokines such as Groα/CXCL1 and ENA-78/CXCL5, whereas no or low suppression was observed in cell expressing the CapΔ5 mutant [deletion of AA 326–350] and CapΔ6 mutant [deletion of AA 326–394]. We further introduced mutations at the serine and threonine sites at 328 [T328A], 330 [S330A] and 346 [T346A], which are susceptible to phosphorylation by SGK1, and also constructed double mutants [T328A, S330A], [T328A, T346A] and [S330A, T346A]. Expression of all these mutants, with the exception of [S330A, T346A], suppressed the production of CXC chemokine to similar levels as their wild-type counterpart. IκBα was found to be specifically phosphorylated by this double mutant [S330A, T346A] and the CapΔ5 mutant at levels comparable to that induced in their wild-type counterpart. Phosphorylation of NDRG1/Cap43 at both serine330 and threonine346 is required for its suppressive action on the NF-κB signaling pathway and CXC chemokine expression in pancreatic cancer cells.



## 1. Introduction

N-myc downstream-regulated gene 1 (NDRG1)/$Ca^{2+}$-associated protein of 43 kDa (Cap43) is identical to the reducing agent and tunicamycin-responsive protein (RTP), reduced in tumor, 42 kDa (rit42), and differentiation-related gene 1 (Drg1) [1]. The NDRG1/Cap43 gene is mapped to human chromosome 8q24 [2], and encodes a protein with a molecular mass of 43 kDa including three tandem GTRSRSHTSE repeats in its COOH-terminal region [3,4]. Expression of NDRG1/Cap43 is highly susceptible to various stimuli, including hypoxia, heavy metals (nickel, calcium, cobalt and iron), phorbol ester, histone deacetylase inhibitors, retinoids and β-mercaptoethanol, and is regulated by oncogenes (N-myc and c-myc), tumor suppressor genes (p53, VHL and PTEN) and the transcriptional factor Egr-1 under hypoxic conditions, suggesting that NDRG1/Cap43 is a stress-inducible protein [1,5–10]. In response to numerous cellular stimuli, NDRG1/Cap43 regulates the cell cycle, cellular differentiation, apoptosis, hypoxia or metal ion sensing, and cell growth both in vitro and in vivo [1].

With regard to the possible role of NDRG1/Cap43 in tumor growth and angiogenesis, we have previously identified it as one of nine genes that are highly expressed in human renal cancer cell carcinoma, and its expression is closely associated with oncosuppressor VHL gene [11,12]. In human pancreatic cancer, overexpression of NDRG1/Cap43 does not affect cell proliferation,

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but markedly suppresses tumor growth in mouse xenograft models [13]. We have recently demonstrated that NDRG1/Cap43 decreases the expression of CXC chemokines (Groα/CXCL1, IL-8/CXCL8 and ENA-78/CXCL5) that are responsible for the recruitment of macrophages and neutrophils, together with suppression of both tumor angiogenesis and growth in mouse xenograft models. This suppression of CXC chemokine production by NDRG1/Cap43 in pancreatic cancer cells occurs through decreased activation of IκBα [14].

The amino acid sequence of NDRG1/Cap43 indicates the presence of a phosphopantetheine attachment site [3] and a prominent α/β hydrolase fold [15]. In addition, based on potentiometric and spectroscopic studies, Zoroddu et al. [16,17] have reported that this COOH-terminal region of NDRG1/Cap43 is important for nickel and copper binding. The amino acid sequence of NDRG1/Cap43 protein also reveals a few putative phosphorylation sites, including those for calcium-calmodulin kinase II, protein kinase A and protein kinase C [18,19]. The amount of phosphorylated NDRG1/Cap43 protein has been shown to decrease when cell proliferation is arrested [18]. The COOH-terminal region of NDRG1/Cap43 protein serves as a substrate for phosphorylation by serum- and glucocorticoid-regulated kinase 1 (SGK1), which then primes it for phosphorylation by glycogen synthase kinase 3β (GSK3β), but it is not a substrate for phosphorylation by protein kinase B/Akt, p70 ribosomal S6 kinase or p90 ribosomal S6 kinase [20]. NDRG1/Cap43 is phosphorylated at threonine (Thr) 328, serine (Ser) 330, Thr346, Thr356 and Thr366 by SGK1, and further phosphorylated at Ser342, Ser352 and Ser362 by GSK3β [20]. In SGK knockout mice, NDRG1/Cap43 is not phosphorylated [20]. However, the physiologic relevance of such NDRG1/Cap43 phosphorylation remains largely unknown.

In this study, we attempted to clarify the domain of NDRG1/Cap43, and also the putative SGK1 site of NDRG1/Cap43 responsible for its suppression of CXC chemokine expression in pancreatic cancer cells. Our results indicated that a NDRG1/Cap43 deletion mutation as well as Ser/Thr mutations at the C-terminal domain lacking phosphorylation sites for SGK1, markedly restored NDRG1/Cap43-dependent suppression of CXC chemokine expression. On the basis of these results, we discuss the possible phosphorylation site that specifically regulates the suppressive effect of NDRG1/Cap43 on CXC chemokine expression by pancreatic cancer cells.

## 2. Materials and methods

### 2.1. Plasmid constructs

Full-length cDNA of human SGK1 were amplified by PCR using the following primer pairs: 5′-ATGACGGTGAAAACTGAGGCTGC TAAGGGC-3′ and 5′-TCAGAGGAAAGAGTCCGTGGGAGGCGC-3′. To obtain Flag-tagged NDRG1/Cap43 (F-Cap), full-length cDNA of human NDRG1/Cap43 were amplified by PCR using the following primer pairs: 5′-CATGTCTCGGGAGATGCAGGATG-3′ and 5′-AGGCCG CCTAGCAGGAGACC-3′. To amplified NDRG1/Cap43 cDNA was ligated into the pGEM-T easy vector (Promega, Madison, WI, USA) and transferred to the p3XFlag-CMV-10 EXPRESSION VECTOR (SIGMA, Saint Louis, Missouri, USA). Flag-Cap43 deletion mutantΔ2 (130–142AA), Δ4 (180–294AA), Δ5 (326–350AA) and Δ6 (326–394AA) were obtained by partial digestion, as appropriate, to delete NDRG1/Cap43 fragment at DraI site and EheI site (Δ2), BglII sites (Δ4), Bsp68I sites (Δ5), Bsp68I sites (Δ6). To obtain cDNA with point mutation of Flag-tagged NDRG1/Cap43, PCR was carried out using the F-Cap vector. Threonine and serine to alanine point mutations were created within NDRG1/Cap43 cDNA by using the following primers: T328A(Mut1), 5′-GCTAGCATGACCCGCCTGATGC GGTCGCGAGCAGCC-3′; S330A(Mut2), 5′-GCTAGCATGACCCGCCTG ATGCGGTCCCGCACAGCTGCAGGGTCC-3′; T346A(Mut3), 5′-GCTAG CATGACCCGCCTGATGCGGTCCCGCACAGCCTCTGGTTCCAGCGTCAC TTCTCTGGATGGCACCCGCAGCCGCTCCCACGCGTCCGAGG-3′; T328A and S330A(Mut4), 5′-GCTAGCATGACCCGCCTGATGCGGTCCCGCGC GGCCGCTGGTTCC,-3′; T328A and T346A(Mut5), 5′-GCTAGCATGA CCCGCCTGATGCGGTCGCGAGCAGCCTCTGGTTCCAGCGTCACTTCTCT GGATGGCACCCGCAGCCGCTCCCACGCGTCCGAGG-3′; S330A and T346A (Mut6), 5′-GCTAGCATGACCCGCCTGATGCGGTCCCGCACAGC TGCAGGGTCCAGCGTCACTTCTCTGGATGGCACCCGCAGCCGCTCCCA CGCGTCCGAGG-3′ and 5′-GGCTGGCAACTAGAAGGCACAGTCG-3′. The products were ligated into pGEM-T easy vector and mutant NDRG1/Cap43 fragments were ligated into the Flag-Cap43Δ5 vector digested by NheI and XbaI. Stable transfected clones were established using G418 selection.

### 2.2. Materials and cell lines

Human pancreatic cancer cell line (MIApaca-2) was purchased and cultured as described previously [13,14]. Cap#11 (NDRG1/Cap43 cDNA transfectant) was established as described previously [13]. MIApaca-2 transfectants (Cap#11, Mock#18, F-Cap#2, CapΔ2#15, CapΔ4#5, CapΔ5#9, CapΔ6#3, Mut1, Mut2, Mut3, Mut4, Mut5 and Mut6) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and G418 in a humidified atmosphere of 5% $CO_2$ at 37 °C. Anti-Cap43 antibody was generated as previously described [12]. Other antibodies were purchased as follows: anti-β-actin antibody and anti-SGK1 antibody were from Abcam; anti-IκBα antibody, anti-p-IκBα antibody, anti-p-NDRG1/Cap43 (Ser330) and anti-p-NDRG1/Cap43 (Thr346) were from Cell Signaling Technology; anti-Flag M2 antibody was from Sigma; anti-HA-peroxidase (3F10) was purchased from Roche Molecular Biochemicals (Mannheim, Germany).

### 2.3. Western blotting

Western blotting was performed as described previously [13]. Cells were rinsed with ice-cold PBS and lysed in buffer A (buffer containing 50 mM Tris–HCl, 350 mM NaCl, 0.1% NP-40, 5 mM EDTA, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin and 1 mM $Na_3VO_4$). Cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and transferred to immobilon membrane (Millipore, Bedford, MA, USA). After transfer, the membrane was incubated with blocking solution followed by primary antibody. Antibody detection was performed using an enhanced chemiluminescence system (GE Healthcare UK Ltd, Little Chalfont, UK). The intensity of the luminescence was quantified using a CCD camera combined with an image analysis system (LAS-1000; Fuji Film, Japan).

### 2.4. Treatment with phosphatase

Whole-cell lysate prepared from MIApaca-2/Cap#11 cells with buffer A without sodium orthovanadate and phenylmethylsulfonyl fluoride was incubated with calf λ phosphatase at 37 °C for 30 min.

### 2.5. Determination of Groα/CXCL1 and ENA-78/CXCL5 by ELISA

The concentration of Groα/CXCL1 and ENA-78/CXCL5 in the condition medium was measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instruction. In brief, 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 cells were incubated for 24 h. Results were normalized for the number of cells.

### 2.6. Statistical analysis

Data are expressed as means ± SD. Comparisons between groups were done Welch's *t*-test or Student's *t*-test. Differences were considered significant at $P < 0.05$.

## 3. Results

### 3.1. Phosphorylation of NDRG1/Cap43 in human pancreatic cancer cells

The amino acid sequence of NDRG1/Cap43 has been reported to bear a phosphopantetheine attachment site, an α/β hydrolase fold and three tandem repeats [15,19], as well as a few sites that are phosphorylated by SGK [20] (Fig. 1A). Western blotting revealed that NDRG1/Cap43 protein had several bands with different mobilities, including bands designated a, b and c (Fig. 1B). Treatment of NDRG1/Cap43 with phosphatase resulted in one higher-mobility NDRG1/Cap43 protein band (c), and complete disappearance of the two lower-mobility bands (a and b) (Fig. 1B). The latter bands were shown to be phosphorylated.

We then examined whether NDRG1/Cap43 protein was phosphorylated in a human pancreatic cancer cell line, MIApaca-2, transfected with NDRG1/Cap43 (Cap#11) when stimulated by exogenous addition of serum. Expression of the lower-mobility band (arrowhead) of NDRG1/Cap43 protein was increased at 3 h, and the higher-mobility band disappeared also at 3 h after serum stimulation. This increased expression of this phosphorylated lower-mobility band of NDRG1/Cap43 was continuously observed during serum stimulation for 24 h (Fig. 1C).

### 3.2. Establishment of cell lines expressing NDRG1/Cap43 deletion mutants and their expression of CXC chemokines

As the COOH-terminal region of NDRG1/Cap43 protein serves as a substrate for phosphorylation by SGK1 (see Fig. 1A), we generated three different NDRG1/Cap43 deletion mutant constructs (Fig. 2A): the CapΔ2 mutant lacking a 12-amino-acid region (residues 130–142) that includes part of the phosphopantetheine attachment site, the CapΔ4 mutant lacking a 114-amino-acid region (residues 180–294) that includes part of the α/β hydrolase fold, the CapΔ5 mutant lacking a 23-amino-acid region (residues 326–350), and the CapΔ6 mutant lacking the C-terminal region (residues 326–394), which includes the putative sites phosphorylated by SGK1 (Fig. 2A). We then investigated whether the CapΔ5 and CapΔ6 mutants were phosphorylated by SGK1. F-Cap, CapΔ5 and CapΔ6 mutant expression vectors were transfected into cancer cells with or without the SGK1 expression vector (Fig. 2B). Phosphorylation of the CapΔ6 mutant by SGK1 was almost completely blocked, whereas phosphorylation of CapΔ5 was only partly diminished in comparison with F-Cap. As shown in Fig. 2C, we have isolated stable transfectants of wild type and various deletion type of NDRG1/Cap43. We found similar protein expression in the F-Cap#2 (wild type) and CapΔ5#9, whereas those of CapΔ2#15, CapΔ4#5 and CapΔ6#3 were less than 20% of that of F-Cap#2. Therefore, we applied more proteins in CapΔ2#15, CapΔ4#5 and CapΔ6#3 transfectants, suggesting that the domains deleted in the CapΔ2, CapΔ4 and CapΔ6 mutants play a role in the stability of the protein. In all six transfectants including F-Cap#2 and the mock transfectant (Mock#18), there was no apparent expression of endogenous NDRG1/Cap43 (data not shown).

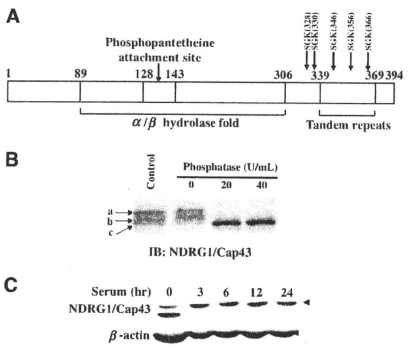


**Fig. 1.** Phosphorylation of NDRG1/Cap43 in MIApaca-2 cells. (A) Positions of the phosphopantetheine attachment site, α/β hydrolase fold, phosphorylation sites by SGK1 and three tandem repeats within NDRG1/Cap43 are shown. (B) Effect of phosphatase on NDRG1/Cap43 phosphorylation. The lysates prepared from Cap#11 cells cultured with 10% serum for 24 h was incubated with calf λ phosphatase and aliquots were collected for Western blot analysis with NDRG1/Cap43 antibody. Each lane shows untreated whole-cell lysate (control), whole-cell lysate incubated at 37 °C for 30 min in the absence and presence (200, 400 and 800 U/mL) of calf λ phosphatase. Band a and b show phosphorylated NDRG1/Cap43 protein, and band c shows dephosphorylated one. (C) Phosphorylation status of NDRG1/Cap43 in MIApaca-2/Cap#11 cells by Western blot analysis. Cap#11 cells were cultured under serum-free conditions, and stimulated with 10% serum for indicated time.

Our previous study showed that overexpression of NDRG1/Cap43 decreased the expression of angiogenic CXC chemokines such as Groα/CXCL1 and ENA-78/CXCL5 in human pancreatic cancer cells [14]. Therefore, we next examined which domain of NDRG1/Cap43 protein is involved in NDRG1/Cap43-dependent suppression of CXC chemokine expression (Fig. 2D). Consistent with our previous study [14], F-Cap#2 cells showed a marked decrease in the production of Groα/CXCL1, ENA-78/CXCL5 in comparison with Mock#18 cells. In both CapΔ2#15 and CapΔ4#5 cells, the expression levels of the CXC chemokines were similar to those in F-Cap#2 cells. However, CapΔ5#9 cells showed similar or higher expression levels of these chemokines as compared with those of Mock#18 cells. Furthermore, in CapΔ6#3 cells, expression levels of Groα/CXCL1 and ENA-78/CXCL5 in ELISA were comparable to those of Mock#18, and significantly higher than those of F-Cap#2 (Fig. 2D). These results suggest that amino acids 326–350 are responsible for NDRG1/Cap43-dependent suppression of the expression of these chemokines.

### 3.3. Serine/threonine phosphorylation of NDRG1/Cap43 by SGK1 is essential for suppression of chemokine expression

Residues $Thr^{328}$, $Ser^{330}$ and $Thr^{346}$ of NDRG1/Cap43 are phosphorylated by SGK1, and CapΔ5 is a mutated form of NDRG1/Cap43 lacking SGK1 phosphorylation sites. We generated various NDRG1/Cap43 constructs bearing mutations within the three Ser/Thr sites that are phosphorylated by SGK1 (Fig. 3A). We established pancreatic cancer cell lines stably expressing these NDRG1/Cap43 point mutations (Fig. 3B) and compared the phosphorylation status at $Ser^{330}$ and $Thr^{346}$ of NDRG1/Cap43 in the various mutant cell lines. Mut2, Mut4 and Mut6, as well as CapΔ5#9, showed decreased phosphorylation of NDRG1/Cap43 ($Ser^{330}$) (Fig. 3B). The phosphorylation of NDRG1/Cap43 ($Thr^{346}$) was apparently decreased in Mut3, Mut5 and Mut6, but not in CapΔ5#9. In CapΔ5#9, this anti-phospho-$Thr^{346}$ antibody also recognized the phosphorylation at $Thr^{356}$ and/or $Thr^{366}$ in tandem repeats, requiring further analysis (data not shown).

We next examined whether these NDRG1/Cap43 mutants showed altered levels of expression of CXC chemokines. Expression of Groα/CXCL1 was suppressed to a similar level by the wild type, Mut1, Mut2, Mut3, Mut4 and Mut5 in comparison with Mock#18

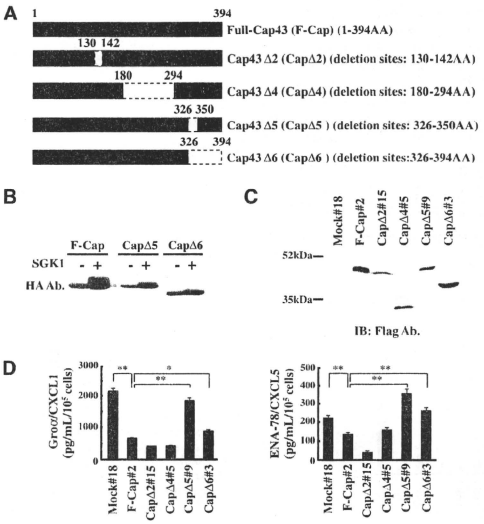


**Fig. 2.** Establishment of cell lines expressing various NDRG1/Cap43 deletion mutants of MIApaca-2. (A) Schema for the NDRG1/Cap43 deletion mutants. (B) Comparison of phosphorylation activity of wild-type (F-Cap) and NDRG1/Cap43 deletion mutants (CapΔ5 and CapΔ6) in the absence or presence of SGK1. PC-3 cells were transfected with F-Cap, CapΔ5 and CapΔ6 expression vectors with or without the SGK expression vector. After 48 h, transfected cells were harvested and subjected to Western blot analysis for HA antibody. (C) Expression of NDRG1/Cap43 deletion mutants in stable transfectants of MIApaca-2. NDRG1/Cap43 deletion mutants were detected by probing with Flag antibody. Protein concentrations of total protein lysates were 5 μg per lane for Mock-18, F-Cap#2 and CapΔ5#9 cells, and was 280 μg per lane for CapΔ2#15, and was 210 μg per lane for CapΔ4#5 cells, and was 30 μg per lane for CapΔ6#3 cells. (D) ELISA analysis of Groα/CXCL1 and ENA-78/CXCL5 in MIApaca-2 transfectants (Mock#18, F-Cap#2, CapΔ2#15, CapΔ4#5, CapΔ5#9 and CapΔ6#3) under 2% serum condition for 24 h. Columns, mean of three independent experiments; bars, SD; $^{*}P < 0.05$; $^{**}P < 0.01$ versus F-Cap#2.

(Fig. 3C). However, Mut6 as well as CapΔ5#9 showed no suppression of Groα/CXCL1 production. Among all the mutant cell lines, only Mut6 showed no suppressive activity on the production of Groα/CXCL1 by pancreatic cancer cells. Expression patterns similar to those in Fig. 3C were observed when other CXC chemokines were examined (data not shown). Furthermore, in culture, these five cell lines showed similar cell growth rates.

### *3.4. Serine/threonine phosphorylation of NDRG1/Cap43 by SGK1 is required for its suppression of NF-κB signal activation*

With regard to the mechanism underlying the suppression of CXC chemokine expression by NDRG1/Cap43, we have reported that reduction of IκBα phosphorylation is intimately involved [14]. We investigated whether NDRG1/Cap43 deletion mutation and point mutation were able to alter the NF-κB signaling pathway. Consistent with our previous study [14], we observed a marked decrease of IκBα phosphorylation in F-Cap#2 cells (Fig. 4A). Phosphorylation of IκBα was increased in Mock#18, CapΔ5#9, Mut4 and Mut6 cells as compared with F-Cap#2. By contrast, the level of IκBα phosphorylation in Mut1, Mut2, Mut3, Mut5 was similar as F-Cap#2 cells (Fig. 4A). To further confirm whether the phosphorylation of NDRG1/Cap43 by SGK1 is specifically responsible for this suppression of IκBα phosphorylation, we examined the effect of SGK1 knockdown on NF-κB signaling. SGK1 knockdown decreased the phosphorylation at both $Ser^{330}$ and $Thr^{346}$ of NDRG1/Cap43 in F-Cap#2 cells (Fig. 4B). This SGK1 knockdown markedly restored the cellular level of p-IκBα in F-Cap#2 cells, in which full NDRG1/Cap43 down-regulated the expression of p-IκBα. By contrast, knockdown of SGK1 in Mock#18 cells did not affect IκBα phosphorylation (Fig. 4B).

## 4. Discussion

In this study, we observed an increase of NDRG1/Cap43 phosphorylation upon stimulation with serum. Also, CapΔ5#9 cells harboring a NDRG1/Cap43 deletion mutant lacking the phosphorylation sites for SGK showed restoration of NDRG1/Cap43-dependent suppression of CXC chemokine expression and IκBα phosphorylation. In CapΔ6#3 cells harboring NDRG1/Cap43 deleting phosphorylation sites for SGK, expression of Groα/CXCL1 and ENA-78/CXCL5 were restored to levels comparable to those of Mock#18 and CapΔ5#9 cells (Fig. 2D). These results strongly indicate that the domain of 326–350 AA is responsible for NDRG1/Cap43-dependent suppression of both CXC chemokine expressions. However, as compared with CapΔ5, in CapΔ6, expression levels of Groα/CXCL1 but not ENA-78/CXCL5 were still lower, suggesting it likely that other unknown domain(s) could also play any role in regulation of their expression.

A NDRG1/Cap43 (S330A and T346A) transfectant (Mut6) did not show suppression of Groα/CXCL1 expression and IκBα phosphorylation. Only the NDRG1/Cap43 (T328A and S330A) transfectant (Mut4) showed restoration of p-IκBα expression and suppression of Groα/CXCL1 expression (Figs. 4A and 3C). At present it remains

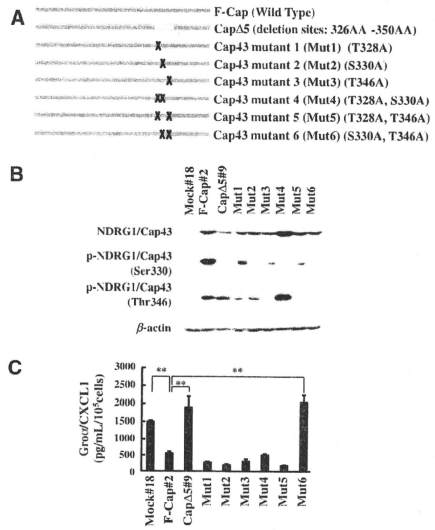


**Fig. 3.** Establishment of NDRG1/Cap43 transfectants harboring point mutations in MIApaca-2. (A) Schema for various NDRG1/Cap43 point mutations. (B) NDRG1/Cap43 expression and its phosphorylation in MIApaca-2 transfectants (Mock#18, F-Cap#2, CapΔ5#9, Mut1, Mut2, Mut3, Mut4, Mut5 and Mut6). (C) ELISA analysis of Groα/CXCL1 protein levels in MIApaca-2 transfectant cells. Columns, mean of three independent experiments; bars, SD. All experiments were done with 2% serum for 24 h. Columns, mean of three independent experiments; bars, SD; $^{**}P < 0.01$ versus F-Cap#2.

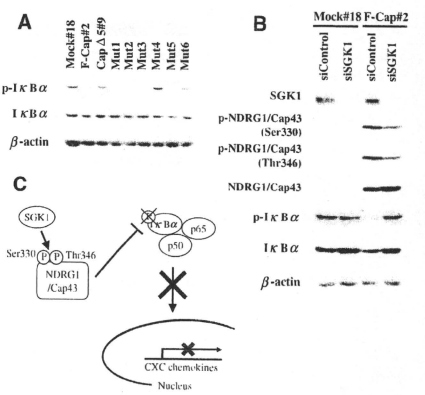


**Fig. 4.** Inhibitory effect of various NDRG1/Cap43 mutants on SGK1-dependent IκBα phosphorylation. (A) IκBα and p-IκBα expression examined by Western blot analysis in MIApaca-2 transfectants of various point mutants. (B) Effect of SGK1 knockdown on expression of phosphorylated NDRG1/Cap43, IκBα and p-IκBα in Mock#18 and F-Cap#2. Western blot analysis of total cell lysates was performed when treated with or without 10 nM SGK1 siRNA for 72 h. (C) A hypothetic model of how NF-κB pathway in pancreatic cancer cells is regulated by NDRG1/Cap43 through SGK1-dependent phosphorylation. In this model, NDRG1/Cap43 phosphorylation at $Ser^{330}$ and $Thr^{346}$ by SGK1 blocks IκBα phosphorylation as well as nuclear translocation of NF-κB (p50/p65), resulting in attenuation of expression of angiogenic CXC chemokine.

unclear why this double mutant (Mut4) restored the expression of p-IκBα, and further analysis of this issue will be required. Furthermore, knockdown of SGK1 expression resulted in restoration of IκBα phosphorylation in F-Cap#2 cells. However, in Mock#18 cells, IκBα phosphorylation was not affected by knockdown of SGK1 expression. Based on these findings, we favor a novel model in which phosphorylation by SGK1 of NDRG1/Cap43 at $Ser^{330}$ and $Thr^{346}$ plays a pivotal role in suppression of the NF-κB signaling pathway and expression of CXC chemokines by NDRG1/Cap43 (Fig. 4C).

The CXC chemokines suppressed by NDRG1/Cap43 have potent chemotactic effects on monocytes/macrophages and neutrophils, and infiltration of these cells, together with neutrophils, promotes angiogenic switching in various cancers, including pancreatic cancer [21–23]. Overexpression of NDRG1/Cap43 has been reported to markedly decrease the infiltration of macrophages and/or neutrophils with suppression of microvascular density and growth in human pancreas cancer xenograft models [14]. We previously reported that in clinical specimens of pancreatic cancer, relatively higher NDRG1/Cap43 expression was significantly correlated with a decrease in the number of infiltrating macrophages and microvascular density [13,14]. Thus, NDRG1/Cap43 might modulate the tumor stroma through suppression of infiltrating inflammatory cells such as macrophages and neutrophils, angiogenesis and tumor growth, possibly through an inflammatory NF-κB signaling pathway.

In our previous study, wild-type NDRG1/Cap43 reduced the expression of IKKβ resulting in down-regulation of the NF-κB signaling pathway in pancreatic cancer cells [14]. IκBα phosphorylation was restored in CapΔ5#9 cells and increased by knockdown of SGK1 (Fig. 4A and B), resulting in enhanced production of angiogenic chemokines and tumor stromal responses including angiogenesis. On the other hand, SGK directly activates IKK to stimulate NF-κB activity in breast cancer cells [24]. NDRG1/Cap43 is phosphorylated by GSK-3, and further phosphorylated by SGK [20]. Also GSK-3 is reportedly involved in constitutive activation of the NF-κB signaling pathway in pancreatic cancer cells [25], and knockdown of the GSK-3 gene decreases IκBα phosphorylation in pancreatic cancer cells [26]. Taken together, the data suggest that the SGK-GSK-NDRG1/Cap43 loop may play key roles in activation or suppression of the NF-κB signaling pathway, affecting tumor stromal responses through altered expression of CXC chemokines. Further study will be required to elucidate the involvement of GSK in the SGK-NDRG1/Cap43-NF-κB loop in pancreatic cancer cells.

In conclusion, our study has demonstrated that phosphorylation of NDRG1/Cap43 at $Ser^{330}$ and $Thr^{346}$ is responsible for its suppressive activity on the NF-κB signaling pathway and CXC chemokine expression. NDRG1/Cap43-dependent modulation of tumor stromal responses such as infiltration of macrophages/neutrophils and tumor angiogenesis could be closely associated with its phosphorylation by SGK1. The phosphorylation status of NDRG1/Cap43 appears to play an important role in the tumor microenvironment, affecting the malignant progression of pancreatic cancer.

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Original contribution

# N-*myc* downstream regulated gene-1/Cap43 may play an important role in malignant progression of prostate cancer, in its close association with E-cadherin ☆,☆☆

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Prostate;
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Prognosis

**Summary** N-*myc* downstream regulated gene-1 (NDRG1)/Cap43 plays an important role in tumor progression and metastases in many kinds of cancers. Recently, it was reported that NDRG1/Cap43 is involved in the aggressiveness of prostate cancer and also that its expression is associated with the expression of E-cadherin in prostate carcinoma cell lines. In the current study, to elucidate the functional and pathologic roles of NDRG1/Cap43 in prostate cancer, we investigated whether the expression of NDRG1/Cap43 is associated with the clinicopathologic parameters of prostate cancer or E-cadherin expression. NDRG1/Cap43 expression and E-cadherin expression were examined immunohistochemically in 148 patients with prostate cancer. We investigated the correlation between membranous or cytoplasmic expression of NDRG1/Cap43 and E-cadherin and evaluated the prognostic or clinicopathologic significance of the expression of NDRG1/Cap43. The patients with decreased NDRG1/Cap43 membranous expression showed significantly lower disease-free survival rates compared with the patients with preserved NDRG1/Cap43 membranous expression. Decreased membranous and high cytoplasmic NDRG1/Cap43 expression was also correlated with a higher Gleason score. A significant correlation was observed between NDRG1/Cap43 membranous expression and E-cadherin membranous expression ($r = 0.7130$; $P < .0001$) and between NDRG1/Cap43 cytoplasmic expression and E-cadherin cytoplasmic expression ($r = 0.5847$; $P < .0001$). Decreased

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NDRG1/Cap43 membranous expression had a significant impact on patient disease-free survival in multivariate analysis ($P$ = .0175). NDRG1/Cap43 could be a novel marker for malignant progression and poor prognosis in prostate cancer, plausibly in its close association with the down-regulation of E-cadherin expression.


## 1. Introduction

Prostate cancer is one of the most commonly diagnosed solid cancers [1]. In spite of advances in the detection and treatment of prostate cancer, the mortality rate remains high because current therapeutic strategies are limited in patients with advanced or recurrent cancer. In patients with clinically localized prostate cancer, surgical and radiation treatments are effective; however, a substantial number of patients will experience recurrent disease [2]. The prognosis of patients with prostate cancer is still difficult to predict, and the emergence of effective new approaches for therapy and prognostic markers will depend on the clarification of the mechanisms involved in the progression of this cancer.

The N-*myc* downstream regulated gene-1 (NDRG1)/Cap43 gene has been identified as a nickel- and calcium-inducible gene [3] that is identical to the differentiation-related gene-1 [4]. The authors of several previous studies have proposed a wide variety of cellular stress response and cell growth regulatory mechanisms that appear to be involved in cellular differentiation [4,5], proliferation and growth arrest [6], DNA damage response [7], and tumor progression and metastasis [8-10].

The expression of NDRG1/Cap43 is often elevated in various types of malignant solid tumors, such as the lung, brain, skin, kidney, liver, and breast [11,12] and endometrial carcinoma [13], compared with their concordant normal tissues. Furthermore, expression of NDRG1/Cap43 was significantly higher in poorly differentiated carcinoma than well-differentiated carcinoma of the colon [14] and liver [15]. However, the authors of other studies have reported that NDRG1/Cap43 expression is augmented in normal cells, as well as well-differentiated cancer cells, and is decreased in poorly differentiated colon, breast, prostate, and pancreatic cancer cells [8,9,16-18]. Several studies have shown that NDRG1/Cap43 expression in cancer cells is a predictive marker of good prognosis in patients with cancers of the prostate, breast, colon, and pancreas [9,10,16,19], whereas its expression is a predictive marker of poor prognosis in patients with liver and cervical cancer [15,20]. In these studies, NDRG expression was observed in the membrane and the cytoplasm. However, they did not elucidate whether the membranous expression or the cytoplasmic expression should be evaluated. Furthermore, it is controversial whether NDRG1/Cap43 protein is up-regulated or down-regulated in tumor progression.

Altered expression of E-cadherin, which plays important roles in cell1 adhesion, is associated with more aggressive biologic behavior in prostate cancer [21-23]. Several authors have suggested that NDRG1/Cap43 expression is often associated with E-cadherin expression [8,24-26]. Although the interaction between NDRG1/Cap43 and E-cadherin in vitro studies involving normal and malignant cell lines is being clarified, surprisingly, little is known about the specific function of NDRG1/Cap43 and the relation between NDRG1/Cap43 and E-cadherin expression in prostate cancer tissue.

In this study, we examined whether NDRG1/Cap43 expression was closely correlated with clinicopathologic characteristics of prostate cancer or E-cadherin expression in prostate cancer patients who received radical prostatectomy.

## 2. Materials and methods

### 2.1. Tissues and clinical data

The subjects were 148 patients, who received radical prostatectomy with no chemotherapy or hormonal therapy before surgery and had enough the carcinoma area for the evaluation of immunohistochemistry, at the Kyushu University Hospital, Fukuoka, Japan, between 1997 and 2006. All patients underwent surgery for clinically localized prostate cancer as determined by preoperative prostate-specific antigen (PSA) concentration, digital rectal examination, and prostate needle biopsy. Detailed clinicopathologic findings of the patients are summarized in Table 1. All prostatectomy specimens were completely reviewed to establish stage and grade of the respective prostate cancers. Slides for this study were prepared from the prostate blocks that contained the largest and representative area of the tumor and adjacent normal epithelium.

Four patients received adjuvant hormonal therapy after surgery. Sixteen patients had less than 2 years of follow-up. We excluded these 20 patients from clinical follow-up data. Clinical follow-up data were available for 128 patients. They were monitored for serum PSA. A PSA level greater than 0.4 ng/mL was defined as *PSA recurrence*. PSA recurrence was found in 20% (26/128) of patients. The median follow-up time of patients still relapse-free at the end of analysis was 49.5 months (mean, 50.0 months).

### 2.2. Immunohistochemistry

Immunohistochemistry was performed using the streptavidin-biotin-peroxidase method with a Histofine streptavidin-

**Table 1** Clinicopathologic parameters in 148 patients with prostate cancer

| | n |
|---|---|
| Age | |
| <70 | 99 |
| ≥70 | 49 |
| Preoperative PSA (ng/mL) | |
| <10.0 | 89 |
| ≥10.0 | 54 |
| Unknown | 5 |
| Gleason score | |
| ≤6 | 47 |
| 7 | 89 |
| ≥8 | 12 |
| Pathologic stage | |
| pT2 | 101 |
| pT3-4 | 47 |
| Surgical margin | |
| Negative | 81 |
| Positive | 67 |
| Lymph node metastasis | |
| Negative | 141 |
| Positive | 4 |
| Unknown | 3 |

biotin-peroxidase kit (Nichirei, Tokyo, Japan). The primary antibodies used in this study were anti-NDRG1/Cap43 antibody (rabbit polyclonal, dilution 1:400) [16,18,20] and anti–E-cadherin antibody (mouse monoclonal, clone 36/E-cadherin, dilution 1:1000; BD Transduction Laboratories, San Jose, CA). The specificity of NDRG1/Cap43 antibody was previously demonstrated by Western blotting [16,18]. Sections, 4 $\mu$m thick, from 10% formalin-fixed, paraffin-embedded material were deparaffinized in xylene and rehydrated through ethanol. Then, endogenous peroxidase activity was blocked by methanol containing 0.3% hydrogen peroxidase for 30 minutes. Antigen retrieval was performed by microwave heating in citrate buffer (pH 6.0) for 20 minutes for both antibodies. After exposure to 10% nonimmunized goat serum (for NDRG1/Cap43) or rabbit serum (for E-cadherin) in phosphate-buffered saline for 10 minutes, the sections were incubated at 4°C overnight with primary antibodies. The sections were then incubated with the second antibody for 20 minutes at room temperature. The reaction products were visualized by diaminobenzidine tetrahydrochloride as a chromogen. Finally, the sections were counterstained with hematoxylin.

## 2.3. Immunohistochemical analysis

To assess the NDRG1/Cap43 and E-cadherin expression, we evaluated membranous, cytoplasmic, and nuclear staining. The intensity of membranous expression of NDRG1/Cap43 and E-cadherin in tumor cells was evaluated as negative, weak, moderate, or strong. We next scored the immunoreactivity of NDRG1/Cap43 and E-cadherin membranous expression by estimating the percentage of strongly positive tumor cells. Membranous expression has been used to evaluate E-cadherin expression in prostate cancer, and decreased membranous expression has been defined as aberrant expression [21,23,27-29]. The authors of some studies reported that the positive cytoplasmic expression of E-cadherin was also included in the aberrant expression [21,27]. Immunoreactivity of NDRG1/Cap43 and E-cadherin cytoplasmic expression was scored using a 4-tier scale by estimating the intensity of labeled tumor cells as previously described [15]. The intensity level was scored as follows: 0, negative (no staining); 1, equivocal; 2, weak positive (weak to moderate intensity in any percentage of tumor cells); and 3, strong positive (strong intensity in at least 30% of tumor cells).

For scoring NDRG1/Cap43 nuclear expression, tumors were considered positive when more than 10% of cells showed nuclear expression.

## 2.4. Statistical analysis

We used $\chi^2$ test and Fisher extract test for statistical analysis of the correlations between immunohistochemical NDRG1/Cap43 expression and the clinicopathologic parameters. Pearson correlation coefficient analysis was applied across the NDRG1/Cap43 and E-cadherin membranous expression, and Spearman rank correlation analysis was applied across the NDRG1/Cap43 cytoplasmic and membranous expression, or the NDRG1/Cap43 cytoplasmic and E-cadherin cytoplasmic expression. Disease-free survival was taken as the period between surgery and the date of the last follow-up or PSA recurrence by disease. Survival curves were calculated by the Kaplan-Meier method, and the significance was analyzed by log-rank test. The Cox proportional hazards model was used for multivariate survival analysis, in which we estimated the following variables: NDRG1/Cap43

**Fig. 1** Immunohistochemical staining of NDRG1/Cap43 and E-cadherin in human prostate tissue. (A) NDRG1/Cap43 expression in normal prostatic epithelium; diffuse and strong membranous and negative cytoplasmic expression was observed in luminal epithelial cells. (B) NDRG1/Cap43 expression in Gleason pattern 3 prostate cancer; diffuse and strong membranous and negative cytoplasmic expression was observed. (C) NDRG1/Cap43 expression in Gleason pattern 5 prostate cancer; decreased membranous and strong cytoplasmic expression was observed. (D) Nuclear expression of NDRG1/Cap43 in mucinous adenocarcinoma (arrow). Cytoplasmic expression was also recognized. (E) E-cadherin expression in normal prostatic epithelium; preserved diffuse membranous E-cadherin expression was observed. (F) E-cadherin expression in Gleason pattern 3 prostate cancer; preserved diffuse membranous expression was observed. (G) E-cadherin expression in Gleason pattern 5 prostate cancer; membranous expression was decreased, and moderate to strong cytoplasmic expression was observed.