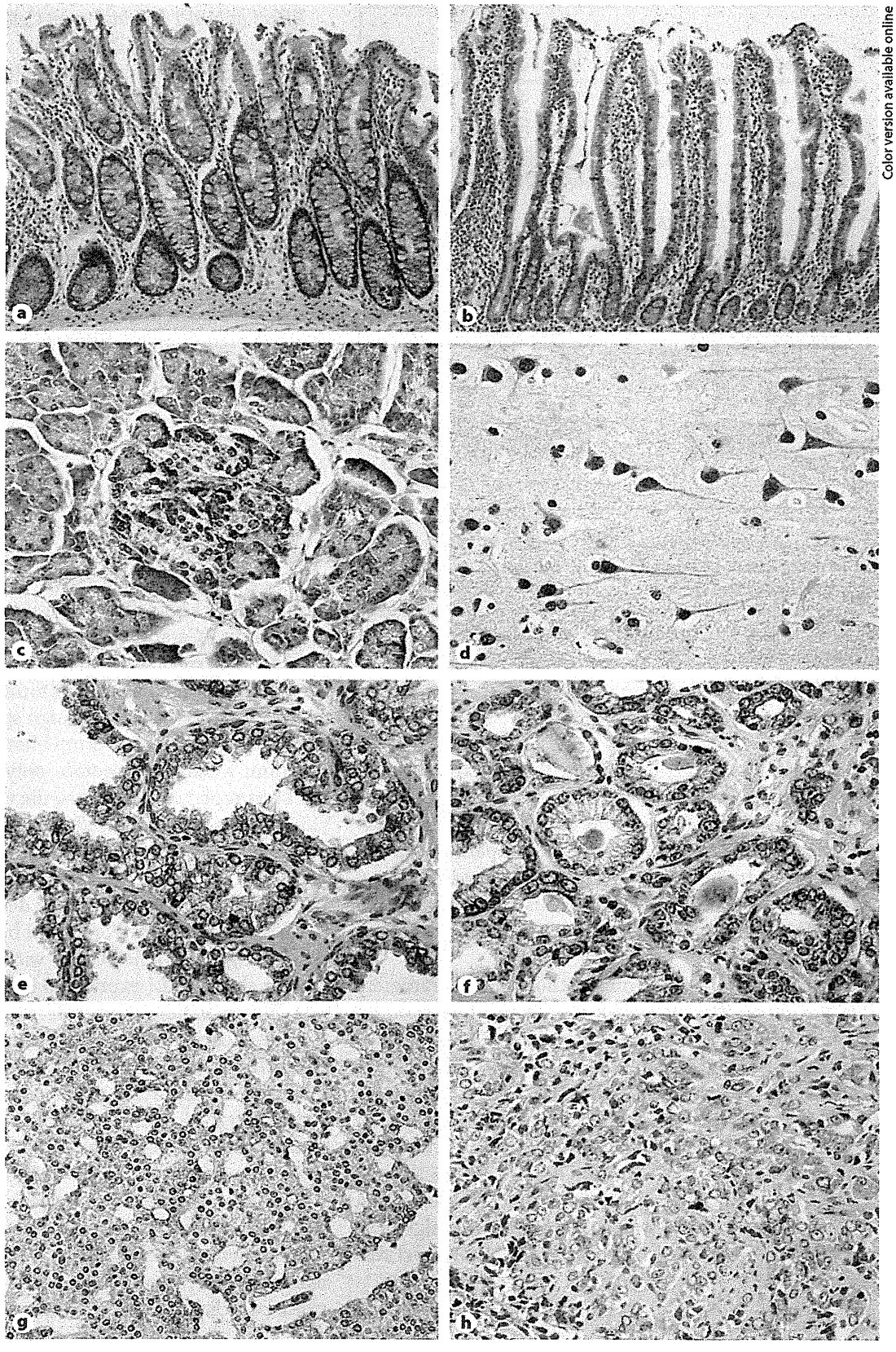

Color version available online

3

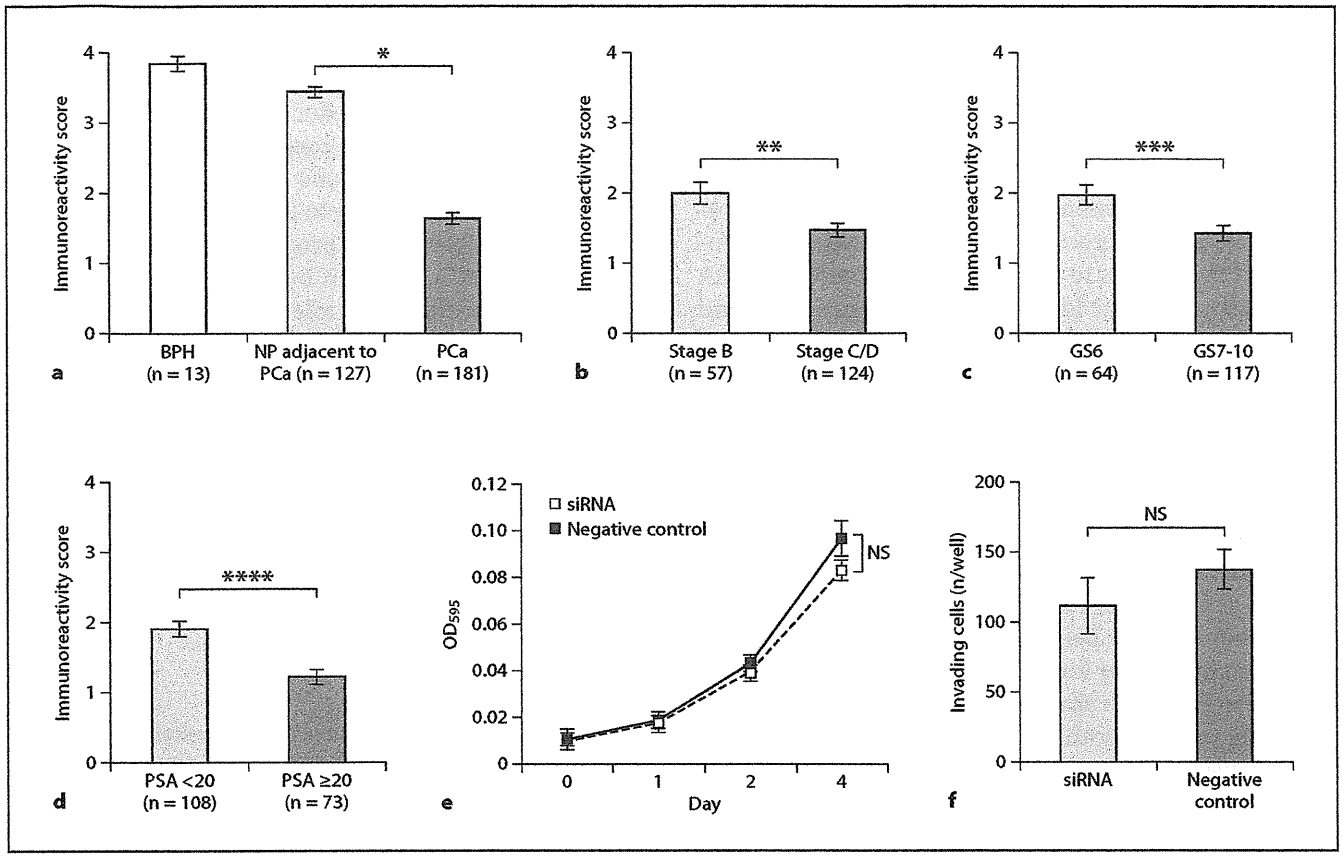

Fig. 4. NBL1 immunoreactivity scores in prostate. NBL1 expression score was higher in NP than in PCa. Means ± SEM. BPH (**a**). NBL1 expression score was higher in stage B than in stages C and D (**b**). NBL1 expression score was higher in PCa with Gleason score 6 than in PCa with Gleason score 7–10 (**c**). NBL1 expression score was higher in PCa with PSA ≤20 than in PCa with PSA >20 (**d**). **e**, **f** Effect of NBL1 knockdown on cell growth (**e**) and cell invasion (**f**) of DU145 cells. **e** Cell growth was assessed by an MTT assay 1, 2 and 4 days after seeding on 96-well plates in DU145 cells. **f** Invading cells were counted after 1 day. Bars and error bars, means and SD of three different experiments. NS = Not significant. * $p < 0.0001$; ** $p = 0.0014$; *** $p = 0.0024$; **** $p < 0.0001$.

and NP. In prostate samples, NBL1 expression was high in NP and lower in PCa. Furthermore, average NBL1 expression was significantly reduced according to the progression of stage, Gleason grade and preoperative PSA value. Because *NBL1* functions as a tumor suppressor gene, these results were consistent with those of previous reports. NBL1 expression was detected in epithelium of the intestine, pancreatic islets and nerve cells, but was absent in other non-cancerous systemic tissues and stromal cells in adult humans. Ozaki et al. [33] examined NBL1 expression in rat tissue by Northern blot analysis and showed that NBL1 was detected in brain, intestine, kidney and lung. They did not test NBL1 expression in rat prostate, but their results were similar to those of our present study in humans.

Fig. 3. Immunohistochemical analysis of NBL1 in non-neoplastic human tissues and prostate tissues. NBL1 expression was detected in the epithelium of the small intestine and colon, islets of the pancreas and nerve cells, respectively (**a–d**). NBL1 staining was observed in the cytoplasm of normal prostate epithelium and PCa cells with Gleason scores 3, 4 and 5, respectively. NBL1 expression was also detected in prostatic ducts (**e–h**).

We also confirmed with Western blot analysis that high NBL1 expression was detected in DU145 cells in culture medium. Nakamura et al. [15] also reported that NBL1 was observed in the culture medium, and the amount of NBL1 secreted from the cells was calculated to be 80% of the total NBL1 protein. Furthermore, NBL1

expression was detected in prostatic ducts in PCa and NP. Therefore, NBL1 may be secreted into semen as well as MSMB, prostatic acid phosphatase and PSA. PSA is produced by secretory epithelial cells in the acini and ducts, and it is secreted directly into the lumen. A characteristic feature of PCa is disruption of the basal cell layer and basement membrane, and this loss of the normal glandular architecture appears to allow PSA increased direct access to the peripheral circulation. PSA is normally found at lower levels in paraurethral and perianal glands, apocrine sweat glands, breast, thyroid and placenta, but these sites do not normally contribute measurable levels of PSA to the circulation [34]. Therefore, in spite of the fact that PSA expression is also higher in NP than in PCa in immunohistochemical analysis, serum PSA is increased in patients with PCa. Although establishment of an enzyme-linked immunosorbent assay system for serum samples is needed to clarify whether NBL1 can serve as a serum marker for detecting and monitoring PCa, we believe that NBL1 might be useful as a serum biomarker for PCa. NBL1 might be beneficial in addition to PSA in situations where PSA is less useful, such as in patients with low PSA level or castration-refractory disease.

The regulation of NBL1 is poorly understood, especially in PCa. Because *NBL1* is thought to be a tumor suppressor gene, it is thought that *NBL1* in PCa has mutations, deletions and methylation. Ozaki et al. [35] reported that two transcription sites were present in the rat NBL1 gene, suggesting the possibility of transcriptional regulation of NBL1. Further studies will clarify how NBL1 is regulated and whether androgen and the androgen receptor axis regulate NBL1. The biological function of the NBL1 protein is also poorly understood in PCa. NBL1 expression correlated with PCa progression, but NBL1 knockdown did not reduce viability and invasiveness relative to the negative control. Because NBL1 was also reported to act as a bone morphogenetic protein (BMP) antagonist by binding to BMPs [36] and BMPs are known to participate in the progression of PCa [37, 38], we speculate that interaction between NBL1 and BMP may play a more important role during growth and development in PCa than in cell cycle inhibition.

In summary, the present study yielded a list of genes that encode secreted proteins present in PCa and NP from CAST analysis. NBL1 expression is narrowly restricted to the prostate and is higher in NP than in PCa. Underexpression of NBL1 is associated with tumor progression. NBL1 has high potential as a biomarker of PCa and its progression.

Acknowledgments

We thank Mr. Shinichi Norimura for his excellent technical assistance and advice. This work was carried out with the kind cooperation of the Research Center for Molecular Medicine, Faculty of Medicine, Hiroshima University. We thank the Analysis Center of Life Science, Hiroshima University, for the provision of their facilities. This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Culture, Science, Sports and Technology of Japan; in part by a Grant-in-Aid for the Third Comprehensive 10-Year Strategy for Cancer Control and for Cancer Research from the Ministry of Health, Labor and Welfare of Japan, and in part by a grant (07-23911) from the Princess Takamatsu Cancer Research Fund.

References

1 Yasui W, Oue N, Kitadai Y, Nakayama H: Recent advances in molecular pathobiology of gastric carcinoma; in Kaminishi M, Takubo K, Mafune K (eds): The Diversity of Gastric Carcinoma Pathogenesis: Diagnosis, and Therapy. Tokyo, Springer, 2005, pp 51–71.
2 Yasui W, Oue N, Ito R, Kuraoka K, Nakayama H: Search for new biomarkers of gastric cancer through serial analysis of gene expression and its clinical implications. Cancer Sci 2004;95:385–392.
3 Hayashi T, Oue N, Sakamoto N, et al: Identification of transmembrane protein in prostate cancer by the *Escherichia coli* ampicillin secretion trap: expression of CDON is involved in tumor cell growth and invasion. Pathobiology 2011;78:277–284.
4 Anami K, Oue N, Noguchi T, et al: Search for transmembrane protein in gastric cancer by the *Escherichia coli* ampicillin secretion trap: expression of DSC2 in gastric cancer with intestinal phenotype. J Pathol 2010;221:275–284.
5 Sirovich BE, Schwartz LM, Woloshin S: Screening men for prostate and colorectal cancer in the United States: does practice reflect the evidence? JAMA 2003;289:1414–1420.
6 Boyle P: Screening for prostate cancer: have you had your cholesterol measured? BJU Int 2003;92:191–199.
7 Carter HB: Prostate cancers in men with low PSA levels – must we find them? N Engl J Med 2004;350:2292–2294.
8 Gambert SR: Prostate cancer. When to offer screening in the primary care setting. Geriatrics 2001;56:22–26.
9 Edwards S, Campbell C, Flohr P, et al: Expression analysis onto microarrays of randomly selected cDNA clones highlights HOXB13 as a marker of human prostate cancer. Br J Cancer 2005;92:376–381.
10 Oefelein MG, Resnick MI: Effective testosterone suppression for patients with prostate cancer: is there a best castration? Urology 2003;62:207–213.
11 Chang SS, Kibel AS: The role of systemic cytotoxic therapy for prostate cancer. BJU Int 2009;103:8–17.

12 Ferguson DA, Muenster MR, Zang Q, et al: Selective identification of secreted and transmembrane breast cancer markers using *Escherichia coli* ampicillin secretion trap. Cancer Res 2005;65:8209–8217.
13 von Heijne G: A new method for predicting signal sequence cleavage sites. Nucleic Acids Res 1986;14:4683–4690.
14 Kadonaga JT, Gautier AE, Straus DR, Charles AD, Edge MD, Knowles JR: The role of the beta-lactamase signal sequence in the secretion of proteins by *Escherichia coli*. J Biol Chem 1984;259:2149–2154.
15 Nakamura Y, Ozaki T, Nakagawara A, Sakiyama S: A product of DAN, a novel candidate tumour suppressor gene, is secreted into culture medium and suppresses DNA synthesis. Eur J Cancer 1997;33:1986–1990.
16 Sobin LH, Wittekind CH (eds): TNM Classification of Malignant Tumors, ed 6. New York, Wiley-Liss, 2002, pp 184–187.
17 Eble JN, Sauter G, Epstein JI, et al: World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Urinary System and Male Genital Organs. Lyon, IARC Press, 2004.
18 Kondo T, Oue N, Yoshida K, et al: Expression of POT1 is associated with tumor stage and telomere length in gastric carcinoma. Cancer Res 2004;64:523–529.
19 Gibson UE, Heid CA, Williams PM: A novel method for real time quantitative RT-PCR. Genome Res 1996;6:995–1001.
20 Yasui W, Ayhan A, Kitadai Y, et al: Increased expression of p34cdc2 and its kinase activity in human gastric and colonic carcinomas. Int J Cancer 1993;53:36–41.
21 Ohtori S, Yamamoto T, Ino H, et al: Differential screening-selected gene aberrative in neuroblastoma protein modulates inflammatory pain in the spinal dorsal horn. Neuroscience 2002;110:579–586.
22 Sakamoto N, Oue N, Noguchi T, et al: Serial analysis of gene expression of esophageal squamous cell carcinoma: ADAMTS16 is upregulated in esophageal squamous cell carcinoma. Cancer Sci 2010;101:1038–1044.
23 Alley MC, Scudiero DA, Monks A, et al: Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Res 1988;48:589–601.
24 Whitaker HC, Warren AY, Eeles R, Kote-Jarai Z, Neal DE: The potential value of microseminoprotein-β as a prostate cancer biomarker and therapeutic target. Prostate 2010;70:333–340.
25 Xu B, Wang J, Tong N, et al: A functional polymorphism in MSMB gene promoter is associated with prostate cancer risk and serum MSMB expression. Prostate 2010;70:1146–1152.
26 Henshall SM, Horvath LG, Quinn DI, et al: Zinc-α_2-glycoprotein expression as a predictor of metastatic prostate cancer following radical prostatectomy. J Natl Cancer Inst 2006;98:1420–1424.
27 Lapointe J, Li C, Higgins JP, et al: Gene expression profiling identifies clinically relevant subtypes of prostate cancer. Proc Natl Acad Sci USA 2004;101:811–816.
28 Asmann YW, Kosari F, Wang K, Cheville JC, Vasmatzis G: Identification of differentially expressed genes in normal and malignant prostate by electronic profiling of expressed sequence tags. Cancer Res 2002;62:3308–3314.
29 Shaikhibrahim Z, Lindstrot A, Buettner R, Wernert N: Analysis of laser-microdissected prostate cancer tissues reveals potential tumor markers. Int J Mol Med 2011;28:605–611.
30 Nakamura Y, Ozaki T, Ichimiya S, Nakagawara A, Sakiyama S: Ectopic expression of DAN enhances the retinoic acid-induced neuronal differentiation in human neuroblastoma cell lines. Biochem Biophys Res Commun 1998;243:722–726.
31 Hanaoka E, Ozaki T, Nakamura Y, Moriya H, Nakagawara A, Sakiyama S: Overexpression of DAN causes a growth suppression in p53-deficient SAOS-2 cells. Biochem Biophys Res Commun 2000;278:20–26.
32 Ozaki T, Nakayama Y, Enomoto H, Hirose M, Sakiyama S: Overexpression of DAN gene product in normal rat fibroblasts causes a retardation of the entry into the S phase. Cancer Res 1995;55:895–900.
33 Ozaki T, Sakiyama S: Molecular cloning and characterization of a cDNA showing negative regulation in v-src-transformed 3Y1 rat fibroblasts. Proc Natl Acad Sci USA 1993;90:2593–2597.
34 Balk SP, Ko YJ, Bubley GJ: Biology of prostate-specific antigen. J Clin Oncol 2003;21:383–391.
35 Ozaki T, Enomoto H, Nakamura Y, et al: The genomic analysis of human DAN gene. DNA Cell Biol 1997;16:1031–1039.
36 Kim AS, Pleasure SJ: Expression of the BMP antagonist Dan during murine forebrain development. Brain Res Dev Brain Res 2003;145:159–162.
37 Hamdy F, Autzen P, Robinson MC, Horne CH, Neal DE, Robson CN: Immunolocalization and messenger RNA expression of bone morphogenetic protein-6 in human benign and malignant prostate tissue. Cancer Res 1997;57:4427–4431.
38 Dai J, Keller J, Zhang J, Lu Y, Yao Z, Keller ET: Bone morphogenetic protein-6 promotes osteoblastic prostate cancer bone metastases through a dual mechanism. Cancer Res 2005;65:8274–8285.

New molecular staging with G-factors (VEGF-C and Reg IV) by supplementing TNM classification in colorectal cancers

TETSUJI SAWADA[1], MASAKAZU YASHIRO[1,2], KAZUHIRO SENTANI[3], NAOHIDE OUE[3], WATARU YASUI[3], KOHJI MIYAZAKI[4], KEITA KAI[5], HIDETO FUJITA[6], KEISHI NAKAMURA[6], KIYOSHI MAEDA[1], YOSHIHIRO KAKEJI[7], SHOJI NATSUGOE[8], KEN SHIRABE[9], SACHIYO NOMURA[10], YUTAKA SHIMADA[11], NAOHIRO TOMITA[12], KOSEI HIRAKAWA[1] and YOSHIHIKO MAEHARA[9]

[1]Department of Surgical Oncology and [2]Oncology Institute of Geriatrics and Medical Science, Osaka City University Graduate School of Medicine, Osaka; [3]Department of Molecular Pathology, Hiroshima University Institute of Biomedical and Health Sciences, Hiroshima; Departments of [4]Surgery, and [5]Pathology and Microbiology, Saga University Faculty of Medicine, Saga; [6]Department of Gastroenterological Surgery, Division of Cancer Medicine, Graduate School of Medical Science, Kanazawa University, Kanazawa; [7]Division of Gastrointestinal Surgery, Department of Surgery, Graduate School of Medicine, Kobe University, Kobe; [8]Department of Surgical Oncology and Digestive Surgery, Kagoshima University School of Medicine, Kagoshima; [9]Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka; [10]Department of Gastrointestinal Surgery, Graduate School of Medicine, University of Tokyo, Tokyo; [11]Department of Surgery and Science, Graduate School of Medicine and Pharmaceutical Sciences for Research, University of Toyama, Toyama; [12]Division of Lower GI, Department of Surgery, Hyogo College of Medicine, Hyogo, Japan

Received August 2, 2013; Accepted September 2, 2013

DOI: 10.3892/or.2013.2787

Abstract. Staging classification of colorectal cancers is performed by the UICC/TNM classification system, which is the global gold standard. However, we often experience in clinical practice that there are considerable differences in prognoses between patients who have the same classification particularly in stage II and III cancers. The aim of this study was to propose a new TNM-G classification to predict prognosis and recurrence by supplementing the conventional TNM classification. A total of 220 cases of colorectal cancer, including 77 at stage II and 143 at stage III, were registered from four independent facilities. Immunohistochemical staining for 7 molecules, such as p53, vascular endothelial growth factor (VEGF)-A, VEGF-C, regenerating islet-derived family, member 4 (Reg IV), olfactomedin 4, Claudin-18 and matrix metalloproteinase-7 (MMP-7), was performed to investigate the correlation between clinicopathological factors and expression of each molecule. Based on the results, no significant correlation was observed between the immunostaining expression of these 7 factors and recurrence in total colorectal cancer. Recurrence in stage II (77 cases) was significantly higher in cases positive for Reg IV expression (P=0.042). On analysis of overall survival (OS) and disease-free survival (DFS), VEGF-C and Reg IV expression had a correlation with poor prognosis, therefore, these factors were selected and applied to G-factor classifications so that cases negative for both could be classified as G0, cases positive for either of the factors could be classified as G1, and cases positive for both factors could be classified as G2. While no significant correlation was observed in the recurrence rates between G0 and G2, OS and DFS in stage II cases were significantly poorer for G2 cases in comparison with G0 or G1 cases. The survival curves of OS and DFS in stage II G2 were similar to that of stage III cases. According to these results, prognosis of VEGF-C/Reg IV both positive G2 cases in stage II colorectal cancer was found to be almost equal to the poor survival in stage III cases, and the advancement of one stage up migration based on G-factors may be supposed to be highly feasible for clinical application. In conclusion, the combination of VEGF-C and Reg IV may be a promising factor for clinical staging to supplement the classical TNM classification system, and it may suggest a good indication of adjuvant chemotherapy for G2 cases in stage II colorectal cancers.

Correspondence to: Dr Tetsuji Sawada, Department of Surgical Oncology, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan
E-mail: t_sawada@osaka-ekisaikai.jp

Key words: colorectal cancer, G-factor, TNM classification, molecular stage, VEGF-C, Reg IV

Introduction

The surgical treatment for colorectal cancer has been established, and the developed operating procedures in primary tumor resection with lymphadenectomy including laparoscopic technique have improved and prolonged the survival of colorectal

cancer patients, particularly in stage II and III cases. To date, staging classification of colorectal cancers is performed by the UICC/TNM classification system (1) which is still used as the global gold standard for the decision in selecting treatment of cancers or predicting parameter for prognoses. However, we often experience in actual clinical practice that there are considerable differences in prognoses between patients who have the same classification particularly in stage III cancers, even though the advanced adjuvant chemotherapy after curative resection has been established. The adjuvant chemotherapy for stage II cases still remains controversial, because its benefit in survival has not been clearly defined. Therefore, the G-Project Committee was established by the Japan Society for Gastroenterological Carcinogenesis at the 2005 annual meeting with an aim of investigating whether a new TNM-G classification can be proposed to predict prognosis and recurrence by supplementing the conventional TNM classification. Gene expression (named as G-factors) which can transmit the molecular biological characteristics, would be included as prognostic factors and new classification of cancers in the TNM classification system. While evaluating the convenience of implementing the TNM-G classification system, it was determined that clinical application of RNA and DNA level analysis of the candidate factors would be challenging. Thus, analysis of protein expression levels by immunohistological staining of resected specimens was chosen for nominating the G-factors because of the relative ease of this method. Here, we conducted a multicenter collaborative study with cases extracted from several facilities.

Materials and methods

Patients. In total, 220 cases of colorectal cancer at stage II (n=77) and stage III (n=143) were registered from four institutions. Of 220 cases, 109 were confirmed postoperative recurrence or death within 5 years, and 111 cases were confirmed as 5-10 year recurrence-free survival. The pathological final stages were managed based on the UICC/TNM classification system (1). The four facilities, Department of Surgical Oncology, Osaka City University Graduate School of Medicine (Osaka, Japan); Department of Gastroenterological Surgery, Kanazawa University (Kanazawa, Japan); Department of Surgery and Science, Graduate School of Medicine, Kyushu University (Fukuoka, Japan); Department of Gastroenterological Surgery, Saga University, Faculty of Medicine (Saga, Japan), were selected to extract clinical cases, and resected specimens to examine evidence based on the correlation of staining outcomes and clinicopathologic factors. This study was conducted after obtaining approval from the society's Ethics Committee at the annual meeting in 2007, and then requesting for approval from the ethics committee of each of the four facilities supplying resected specimens. Each facility provided samples according to an implementation planning report. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki (1975). Upon gaining approval from the ethics committees, tissue samples were obtained from each specimen of the most recent cases from each facility, along with anonymous background data such as age, gender, occupation, operative procedure, degree of penetration into the wall (pT), lymph node metastasis (pN), final stage, ly and v factors, histological type, presence or absence of adjuvant therapy and regime, recurrence type, treatment after recurrence, postoperative disease-free survival (DFS), and postoperative overall survival (OS) period. The data and clinicopathological background factors were subsequently analyzed by the Department of Oncology at the Institute of Geriatrics and Medical Science, Graduate School of Medicine, Osaka City University.

Table I. Prognostic factors in colorectal cancer.

Category	Molecules	No. of papers by multivariate (M) or univariate (U) analysis
Oncogene	*k-ras*	
	c-erbB-2	
Tumor suppressor gene	*P53*	M, 6[a]; U, 5[b]
	DCC	
	SPN	
MSI (MMR gene)	MSI/BAT	M, 4[a]; U, 8[b]
Cell proliferation	Polyamine	
Growth factor/ cytokine and those receptor	VEGF	M, 2[a]; U, 6[b]
	IL6	
	IGF	
	cMet	
	EGFR	
Apoptosis signal pathway	TRAIL	
Cell invasion and adhesion	MRP-1	
	uPA	
	Matrilysin	
	S100A4	
	Angiomodulin	
	CD44	M, 1[a]; U, 3[b]
	Laminin	
	β6-integrin	
	α3-integrin	
Angiogenesis	CD105	
Others	Vascular density	M, 3[a]; U, 2[b]
	Galectin	
	CD95	
	Telomerase	

[a]No. of papers by multivariate analysis; [b]no. of papers by univariate analysis. The prognostic factors reported in the 396 published articles between 1990-2005.

Selection for factor analysis and case extraction. As a preliminary step, a literature search of articles published between 1990 and 2005 was conducted in PubMed using the key word 'colorectal cancer' and 'independent prognostic factors'. A total of 396 articles on colorectal cancer, were extracted and reviewed (Table I). The reports indicated 30 molecules as prognostic

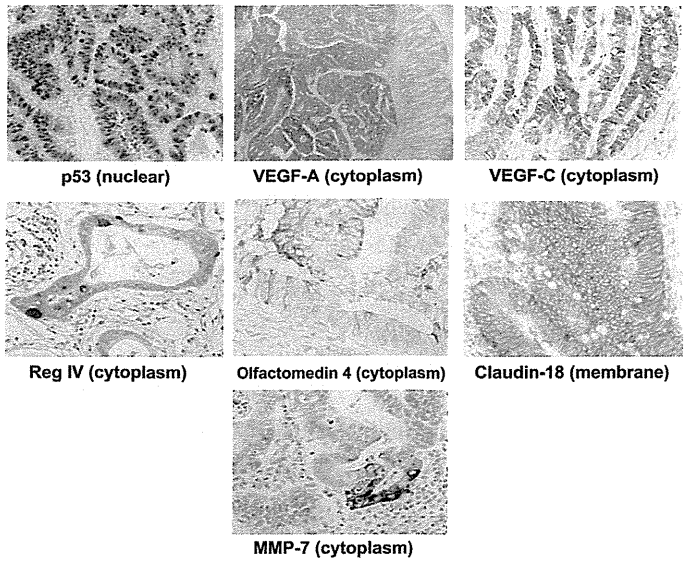

Figure 1. Immunohistochemical determination of p53, VEGF-A, VEGF-C, Reg IV, olfactomedin 4, Claudin-18 and MMP-7. p53 was found in the nuclei of cancer cells. Claudin-18 was observed at cell-cell boundaries of cancer cells. Other molecules were found in the cytoplasm of cancer cells.

factors in colorectal cancer. These were classified into 9 groups based on molecular function: oncogenes, tumor suppressor genes, microsatellite instability, cell proliferation, growth factors/cytokines and their receptors, apoptosis signaling pathways, cell adhesion and invasion, angiogenesis, and others. Concerning the literature search, highly reported prognostic factors in colorectal cancers are: p53, 11 papers; microsatellite instability (MSI), 12; vascular endothelial growth factor (VEGF), 8; vascular density, 5; and CD44, 4. Based on these results, p53 and VEGF are the most common in colorectal cancers and supposed as candidate molecular factors namely 'G factors'. Therefore, three factors, p53, VEGF-A and VEGF-C, were nominated as candidate factors (2-9) and evaluated in stage II and III cancers. In addition to the three factors, five molecules, regenerating islet-derived family, member 4 (Reg IV) (10,11), olfactomedin 4 (12), Claudin-18 (Invitrogen) (13) and matrix metalloproteinase-7 (MMP-7) (14), were added as candidate factors and evaluated.

Methods of immunohistochemical staining and evaluation. In total, 110 cases of colorectal cancer, in which postoperative recurrence/death was confirmed within 5 years, and similarly, 110 cases, in which 5-10 year recurrence-free survival was confirmed, were used in a case-control study. A total of 220 patients who had undergone a R0 resection of the primary tumor and were confirmed histologically to have colorectal cancer, were enrolled in this study. Of the 220 cancers, 60 cases were from Osaka City University, 40 from Kanazawa University, 40 from Kyushu University, and 80 were from Saga University. The pathological final stages were managed based on Japanese Classification of Colorectal Carcinoma (7th edition) (15) which was revised based on the UICC/TNM Classification of malignant tumors (1). The above four institutions ultimately registered 220 cases of colorectal cancer (111 recurrence-free and 109 with recurrence), and these specimens were formalin-fixed and paraffin-embedded. Immunohistochemical staining was performed at the Department of Molecular Pathology at Hiroshima University (Hiroshima, Japan) using seven primary antibodies for p53 (DO-7; Dako), VEGF-A (Santa Cruz Biotechnologies, Inc.), and VEGF-C (American Research Products, Inc.), Reg IV; olfactomedin 4; Claudin-18 (Invitrogen); and MMP-7 (141-7B2; Daiichi Fine Chemicals, Inc.). Paraffin-embedded specimens were sectioned at 4 μm, hydrophilized, and microwaved for 30 min in pH 6.0 citric acid buffer or autoclaved in ethylenediaminetetraacetic acid buffer to activate the antigen. Intrinsic peroxidase was deactivated by incubation with 3% H_2O_2 for 10 min, and blocking was performed using sheep serum and reacting with each primary antibody for 1 h at room temperature. The samples were incubated in diaminobenzidine solution for 10 min, and counterstained with hematoxylin. The stained area was scored by the percentage of immunopositive cells as an index of the expression of each molecule. Cases that were not at all stained were scored as 0, cases with <10% of stained tumor cells were 1+, cases with 10-50% of stained tumor cells were 2+, and cases with >50% of stained tumor cells were 3+. Evaluation of immunostaining was conducted independently by two pathologists, and any discrepancies in assessment were discussed and reassessed by microscopy.

Data analysis and testing for significant difference. The correlation between a clinicopathological factor and immunostaining result was analyzed by the Chi-square test or Fisher's exact test. The survival duration was calculated using the Kaplan-Meier method and analyzed by the log-rank test to compare the cumulative survival durations in the patient groups. In all tests, a P-value of <0.05 was considered to represent statistical significance. SPSS statistical software (SPSS Japan Inc., Tokyo, Japan) was used for all analyses.

Results

Positive staining rate in colorectal cancer. Cases with >10% of stained tumor cells and scoring 2 or 3+ were assessed to be positive by two independent pathologists. Each representative

Table II. Correlation between postoperative recurrence and clinicopathological features in 220 patients with colorectal cancer.

Clinicopathological factors	Recurrence		P-value
	Negative n=111 (50%)	Positive n=109 (50%)	
Gender			
Male	59 (47)	66 (53)	0.268
Female	52 (55)	43 (45)	
Location			
Right	31 (54)	26 (46)	0.465
Left	79 (49)	83 (51)	
pT			
1	2 (100)	0 (0)	0.008
2	5 (50)	5 (50)	
3	96 (53)	84 (47)	
4	8 (29)	20 (71)	
pN			
Negative	48 (59)	33 (41)	0.046
Positive	63 (45)	76 (55)	
Stage			
II	46 (60)	31 (40)	0.043
III	65 (46)	78 (54)	
Histologic type			
Diffuse	11 (38)	18 (62)	0.140
Intestinal	100 (53)	90 (47)	
Lymphatic invasion			
Negative	53 (60)	35 (40)	0.041
Positive	58 (44)	73 (56)	
Venous invasion			
Negative	74 (52)	68 (48)	0.509
Positive	37 (48)	40 (52)	
p53			
Negative	51 (48)	55 (52)	0.503
Positive	60 (53)	54 (47)	
VEGF-A			
Negative	46 (51)	44 (49)	0.871
Positive	65 (50)	65 (50)	
VEGF-C			
Negative	46 (52)	42 (48)	0.660
Positive	65 (49)	67 (51)	
Reg IV			
Negative	91 (52)	83 (48)	0.287
Positive	20 (44)	26 (56)	
Olfactomedin 4			
Negative	43 (52)	40 (48)	0.755
Positive	68 (50)	69 (50)	
Claudin18			
Negative	104 (52)	98 (48)	0.306
Positive	7 (39)	11 (61)	
MMP-7			
Negative	58 (48)	62 (52)	0.491
Positive	53 (53)	47 (47)	

positive expression in histological image for colorectal cancer is depicted in Fig. 1. Concerning to the positive staining rate of each factor, 51.8% of p53, 59.1% of VEGF-A, 60.0% of VEGF-C, 20.9% of Reg IV, 62.3% of olfactomedin 4, 8.2% of Claudin-18 and 45.5% of MMP-7 were positive in colorectal cancers (Table II).

Correlation of postoperative recurrence and clinicopathological factors or the candidate molecular factors in colorectal cancer. Examination of the 220 colorectal cancer cases revealed a significant correlation between the postoperative recurrence and pT stage (P=0.008), pN stage (P=0.046), clinical stage (P=0.043), and ly factors (P=0.041), whereas no significant correlation was observed between the presence or absence of expression of the seven molecular factors and recurrence (Table II). Analysis of each stage revealed that the postoperative recurrence was significantly higher in Reg IV positive cases (P=0.042) at stage II in compared to negative cases, while no significant correlation was observed for any of the factors in stage III (Table III).

Prognostic analysis of OS and DFS in expression of the candidate molecular factors of colorectal cancer. In OS of stage II and III, colorectal cancer cases positive for VEGF-C and Reg IV tended to have poorer OS in comparison with the negative cases, although this was not significant. The prognosis of OS was significantly poorer (P=0.036) in stage II cases positive for VEGF-C expression in comparison with VEGF-C negative cases, moreover positive cases for Reg IV in stage II demonstrated significant poorer prognosis (P=0.0022) compared to negative cases. Reg IV positive cases at stage II and VEGF-C positive cases at stage III tended to have poorer DFS (P=0.052 and 0.094, respectively) (Fig. 2). In contrast, no significant difference was observed in OS between positive and negative cases for any of the 7 factors at stage III. Also, no significant difference of DFS was found between positive and negative groups in stage II cases.

Feasibility of the candidate molecular factors. According to the above results, we selected VEGF-C and Reg IV as nominating factors in colorectal cancer. We then analyzed the relationship between the combination of VEGF-C and Reg IV expression and prognosis. Then, colorectal cancer patients were divided into three groups based on the VEGF-C and Reg IV expression; G0 group (both negative group, n=69), G1 group (either positive group, n=104), G2 group (both positive group, n=37). Fig. 3 shows the relationship between the combination

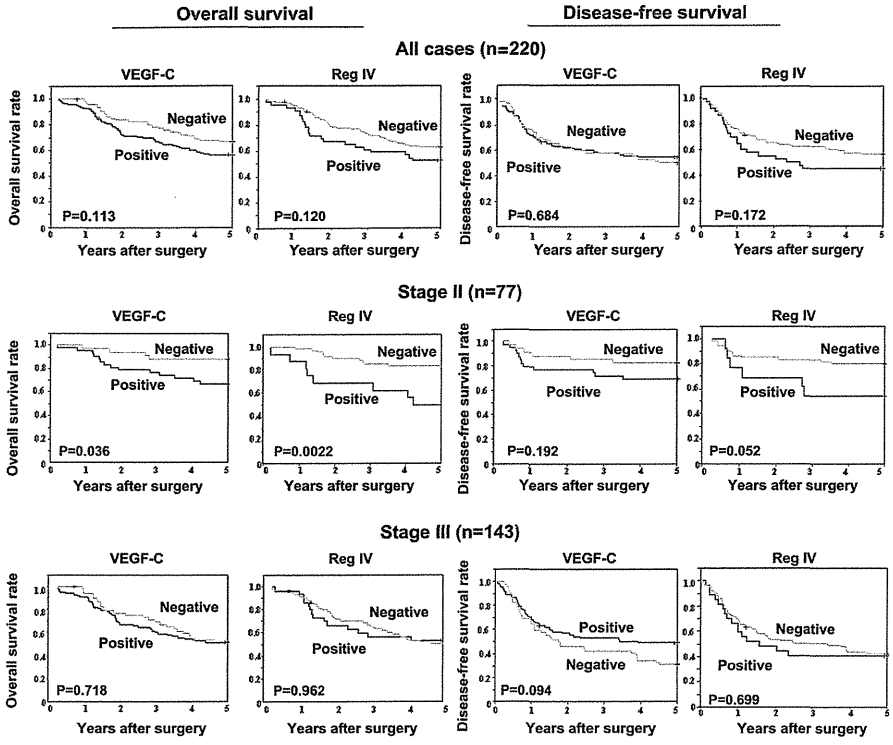

Figure 2. The overall (OS) and disease-free survival (DFS) curves of 220 colorectal cancer cases. Colorectal cancer with VEGF-C-positive expression showed a significantly worse OS time (P=0.036), and cases with Reg IV expression tend to have the worst OS time (P=0.0022).

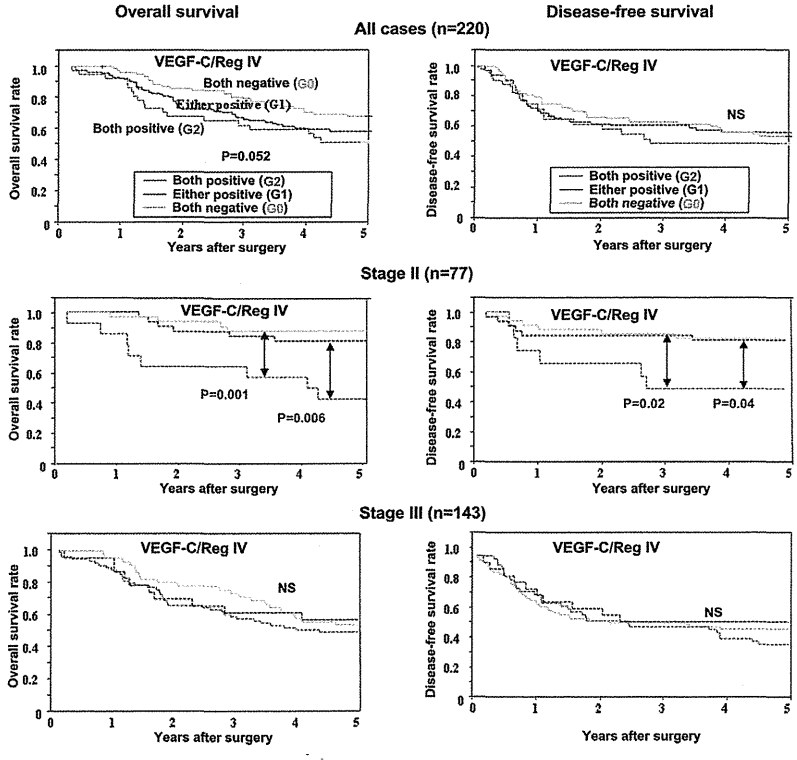

Figure 3. Survival of patients with colorectal cancer based on VEGF-C and Reg IV expression. In patients at stage II, the overall survival (OS) of the combination of VEGF-C and Reg IV positive group (G2) was significantly poorer than that of the combination of either positive (G1) and both negative group (G0) (P=0.006 and 0.001, respectively). The disease-free survival (DFS) of the combination of both VEGF-C and Reg IV positive group (G2) was significantly poorer than that of either positive (G1) and both negative group (G0) (P=0.02 and 0.04, respectively). In contrast, no significant difference of OS or DFS was observed among G0, G1, and G2 groups in all of cases or in stage III cases.

of VEGF-C and Reg IV expression and prognosis. In stage II cases, OS of G2 cases were significantly poorer in comparison with that of G0 cases (P=0.001) and G1 cases (P=0.006), and DFS was also poorer than that of G0 cases (P=0.02) and G1

Table III. Correlation between postoperative recurrence and candidate G-factors in 220 patients with colorectal carcinoma at stage II and III.

	Stage II (n=77) Recurrence			Stage III (n=143) Recurrence		
Clinicopathological factors	Negative n=46 (60%)	Positive n=31 (40%)	P-value	Negative n=65 (45%)	Positive n=78 (55%)	P-value
p53						
Negative	17 (53)	15 (47)	0.318	34 (46)	40 (54)	0.903
Positive	29 (64)	16 (36)		31 (45)	38 (55)	
VEGF-A						
Negative	18 (60)	12 (40)	0.970	28 (47)	32 (53)	0.805
Positive	28 (60)	19 (40)		37 (45)	46 (55)	
VEGF-C						
Negative	21 (62)	13 (38)	0.747	25 (46)	29 (54)	0.875
Positive	25 (58)	18 (42)		40 (45)	49 (55)	
Reg IV						
Negative	40 (66)	21 (34)	0.042	51 (45)	62 (55)	0.881
Positive	6 (38)	10 (62)		14 (47)	16 (53)	
Olfactomedin 4						
Negative	19 (68)	9 (32)	0.272	24 (44)	31 (56)	0.730
Positive	27 (55)	22 (45)		41 (47)	47 (53)	
Claudin-18						
Negative	43 (61)	28 (39)	0.612	61 (47)	70 (53)	0.378
Positive	3 (50)	3 (50)		4 (33)	8 (67)	
MMP-7						
Negative	23 (55)	19 (45)	0.329	35 (45)	43 (55)	0.878
Positive	23 (66)	12 (34)		30 (46)	35 (54)	

Table IV. Relationship between reccurrence and two molecular factors.

	Recurrence, n (%)		
VEGF-C and Reg IV	Negative	Positive	P-value
Total (n=220)	111 (51)	109 (49)	
Both negative G0 (n=79)	41 (52)	38 (48)	
Either positive G1 (n=104)	55 (53)	49 (47)	
Both positive G2 (n=37)	15 (41)	22 (59)	0.413
Stage II (n=77)	46 (60)	31 (40)	
Both negative G0 (n=32)	20 (63)	12 (37)	
Either positive G1 (n=31)	21 (68)	10 (32)	
Both positive G2 (n=14)	5 (36)	9 (64)	0.117
Stage III (n=143)	65 (45)	78 (55)	
Both negative G0 (n=47)	21 (45)	26 (55)	
Either positive G1 (n=73)	34 (47)	39 (53)	
Both positive G2 (n=23)	10 (43)	13 (57)	0.959

cases (P=0.04). In contrast, no significant difference of OS or DFS was observed among G0, G1 and G2 groups in all of cases or in stage III cases (Fig. 3). Table IV shows the relationship between the combination of VEGF-C and Reg IV expression

and the postoperative recurrence. In all 220 colorectal cancer cases, the recurrence rate was slightly higher (59%) in G2 cases compared to 48% in G0 cases while no significant difference was observed (P=0.413). In stage II cases, the recurrence rate of G2 cases (64%) was high in comparison with that of G1 cases and G0 cases (32 and 37%, respectively), while the difference was not significant (P=0.117).

Discussion

This study was a multicenter collaborative study with cases extracted from four universities. The four institutions ultimately registered 220 cases of colorectal cancer. The analysis of protein expression levels by immunohistological staining was selected for nominating the G-factors. The immunohistological staining was performed in each Department of Pathology by keeping patient infomation and clinicopathologic factors anonymous. The relationship between protein expression levels and clinicopathological background factors was independently analyzed at each facility. Therefore, the objectivity of these results can be considered to have high reliability and authenticity.

Precise clinical classification of prognosis might be useful to select a strategy for rigorous adjuvant chemotherapy and careful follow-up (16,17). The present study was conducted to establish a new classification system based on the biochemical characteristics of cancer, which would supplement the conventional TNM staging system. As shown in Fig. 2, VEGF-C and Reg IV expression was associated with a significantly poorer prognosis for OS of stage II colorectal cancer. However, these factors alone could not be found in the progressing stage to advance. Concerning these results, single use of G-factors by supplementing TNM staging may be difficult and limited. Because TNM staging is classified into T1-4, N0-3, and M0-1, consequently, in regard to the feasibility of TNM-G staging, combination of VEGF-C and Reg IV in 7 factors were selected and analyzed for usefulness. High frequent recurrences in stage II cases were observed in both positive cases (G2), but no relationship of recurrence was found among the 3 groups, G0-2. In contrast, a significant difference in OS was observed between G0/1 and G2 in stage II cases. Furthermore, the prognosis of OS and DFS of stage II G2 cases showed a survival curve apparently similar to that of stage III cases. This result indicated that prognosis of VEGF-C and Reg IV both positive G2 in stage II colorectal cancer was the same as that of stage III cases, and the advancement of one stage up based on G-factors may be highly feasible for clinical application. These findings suggested that TNM-G staging may have a possibility for use as a reasonable supplement to the TNM classification system.

Through the collaboration of many facilities and the Japan Society for Gastroenterological Carcinogenesis, the present study was schemed to investigate the feasibility of new molecular staging as a G-factor to further supplement the TNM classification system, which is the standard staging system used for colorectal cancers. With the advancement of molecular-targeting drugs, we investigated the possible application of G-factors, which are derived from molecular biological characteristics of cancer, in staging along with clinicopathological factors. However, in accordance with previous reports from single institutions, no correlation between recurrence/prognosis and up-staging migration was found. Thus, the proposal of an individual single G-factor was supposed to be challenging. However, when colorectal cancer was limited to stage II, the present results indicated that G2 cases both positive for VEGF-C/Reg IV were likely to advance up to stage III, suggesting that G-factors can be used to supplement initial staging by TNM classification. Application and effect of adjuvant chemotherapy for stage II colorectal cancers still remains controversial. The present results may suggest a good indication of adjuvant chemotherapy for G2 cases in stage II. In future studies, the highly relevant factors may be identified by the involvement of the degree of molecular biological malignancy to establish TNM-G staging, and application of these factors by supplementing TNM classification may contribute to more accurate prediction of prognosis.

In conclusion, the members of the Japan Society for Gastroenterological Carcinogenesis, investigated the feasibility of a new molecular factor(s) to further supplement the TNM classification system, and found that the combination of VEGF-C and Reg IV might be a promising factor for clinical staging to supplement the classical TNM classification system, and it may suggest a good indication of adjuvant chemotherapy for G2 cases in stage II colorectal cancers.

Acknowledgements

We thank Emeritus Professor Eiichi Tahara (Hiroshima Cancer Seminar Foundation, Hiroshima, Japan) and Emeritus Professor Michio Kaminishi (Showa General Hospital, Tokyo, Japan) for helpful advice. This study is partially funded by the Japan Society for Gastroenterological Carcinogenesis.

References

1. Sobin LH: TNM, sixth edition: new developments in general concepts and rules. Semin Surg Oncol 21: 19-22, 2003.
2. Tanigawa N, Amaya H, Matsumura M and Shimomatsuya T: Correlation between expression of vascular endothelial growth factor and tumor vascularity, and patient outcome in human gastric carcinoma. J Clin Oncol 15: 826-832, 1997.
3. Maeda K, Kang SM, Onoda N, *et al*: Expression of p53 and vascular endothelial growth factor associated with tumor angiogenesis and prognosis in gastric cancer. Oncology 55: 594-599, 1998.
4. Maehara Y, Tomoda M, Hasuda S, *et al*: Prognostic value of p53 protein expression for patients with gastric cancer - a multivariate analysis. Br J Cancer 79: 1255-1261, 1999.
5. Kakeji Y, Koga T, Sumiyoshi Y, *et al*: Clinical significance of vascular endothelial growth factor expression in gastric cancer. J Exp Clin Cancer Res 21: 125-129, 2002.
6. Fondevila C, Metges JP, Fuster J, *et al*: p53 and VEGF expression are independent predictors of tumour recurrence and survival following curative resection of gastric cancer. Br J Cancer 90: 206-215, 2004.
7. Resnick MB, Routhier J, Konkin T, Sabo E and Pricolo VE: Epidermal growth factor receptor, c-MET, beta-catenin, and p53 expression as prognostic indicators in stage II colon cancer: a tissue microarray study. Clin Cancer Res 10: 3069-3075, 2004.
8. White JD, Hewett PW, Kosuge D, *et al*: Vascular endothelial growth factor-D expression is an independent prognostic marker for survival in colorectal carcinoma. Cancer Res 62: 1669-1675, 2002.
9. Des Guetz G, Uzzan B, Nicolas P, *et al*: Microvessel density and VEGF expression are prognostic factors in colorectal cancer. Meta-analysis of the literature. Br J Cancer 94: 1823-1832, 2006.
10. Oue N, Kuniyasu H, Noguchi T, *et al*: Serum concentration of Reg IV in patients with colorectal cancer: overexpression and high serum levels of Reg IV are associated with liver metastasis. Oncology 72: 371-380, 2007.

11. Oue N, Mitani Y, Aung PP, *et al*: Expression and localization of Reg IV in human neoplastic and non-neoplastic tissues: Reg IV expression is associated with intestinal and neuroendocrine differentiation in gastric adenocarcinoma. J Pathol 207: 185-198, 2005.
12. Oue N, Sentani K, Noguchi T, *et al*: Serum olfactomedin 4 (GW112, hGC-1) in combination with Reg IV is a highly sensitive biomarker for gastric cancer patients. Int J Cancer 125: 2383-2392, 2009.
13. Sanada Y, Oue N, Mitani Y, Yoshida K, Nakayama H and Yasui W: Down-regulation of the claudin-18 gene, identified through serial analysis of gene expression data analysis, in gastric cancer with an intestinal phenotype. J Pathol 208: 633-642, 2006.
14. Ajisaka H, Yonemura Y and Miwa K: Correlation of lymph node metastases and expression of matrix metalloproteinase-7 in patients with gastric cancer. Hepatogastroenterology 51: 900-905, 2004.
15. Japanese Society for Cancer of the Colon and Rectum: Japanese Classification of Colorectal Carcinoma. 7th edition. Kanehara, Tokyo, 2009.
16. Mejia A, Schulz S, Hyslop T, Weinberg DS and Waldman SA: Molecular staging individualizing cancer management. J Surg Oncol 105: 468-474, 2012.
17. Eschrich S, Yang I, Bloom G, *et al*: Molecular staging for survival prediction of colorectal cancer patients. J Clin Oncol 23: 3526-3535, 2005.

Cancer Science
Japanese Cancer Association JCA
Open Access

NRD1, which encodes nardilysin protein, promotes esophageal cancer cell invasion through induction of MMP2 and MMP3 expression

Naohiro Uraoka,[1] Naohide Oue,[1] Naoya Sakamoto,[1] Kazuhiro Sentani,[1] Htoo Zarni Oo,[1] Yutaka Naito,[1] Tsuyoshi Noguchi[2] and Wataru Yasui[1]

[1]Department of Molecular Pathology, Hiroshima University Institute of Biomedical and Health Sciences, Hiroshima; [2]Department of Gastroenterological Surgery, Faculty of Medicine, Oita University, Yufu, Japan

Key words
Esophageal cancer, invasion, nardilysin, NRD1, prognosis

Correspondence
Wataru Yasui, Department of Molecular Pathology, Hiroshima University Institute of Biomedical and Health Sciences, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan.
Tel: +81-82-257-5145; Fax: +81-82-257-5149;
E-mail: wyasui@hiroshima-u.ac.jp

Funding information
Ministry of Education, Culture, Science, Sports and Technology of Japan. Ministry of Health, Labor and Welfare of Japan. The National Institute of Biomedical Innovation (Program for Promotion of Fundamental Studies in Health Sciences). National Cancer Center Research and Development Fund.

Received June 3, 2013; Revised October 16, 2013; Accepted October 27, 2013

Cancer Sci **105** (2014) 134–140

doi: 10.1111/cas.12316

Esophageal squamous cell carcinoma (ESCC) is one of the most common malignancies worldwide. In the present study, to identify novel prognostic markers or therapeutic targets for ESCC, we reviewed a list of genes with upregulated expression in ESCC compared with normal esophagus, as identified by our serial analysis of gene expression (SAGE) analysis. We focused on the *NRD1* gene, which encodes the nardilysin protein. Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) in 34 ESCC tissue samples revealed that mRNA expression of *NRD1* was upregulated in 56% of ESCC tissue samples. Immunohistochemical analysis of nardilysin in 109 ESCC tissue samples demonstrated that 43 (39%) ESCC cases were positive for nardilysin. Nardilysin-positive ESCC cases were more advanced in terms of T classification ($P = 0.0007$), N classification ($P = 0.0164$), and tumor stage ($P < 0.0001$) than nardilysin-negative ESCC cases. Furthermore, nardilysin expression was significantly associated with poorer prognosis ($P = 0.0258$). Univariate and multivariate analyses revealed that nardilysin expression is an independent prognostic classifier of patients with ESCC. The invasiveness of *NRD1*-knockdown TE1 and TE5 esophageal cancer cell lines was less than that of the negative control siRNA-transfected cell lines. Expression of *MMP2* and *MMP3* mRNA was significantly lower in *NRD1*-knockdown TE5 cells than in negative control siRNA-transfected cells. These results suggest that nardilysin is involved in tumor progression, and is an independent prognostic classifier in patients with ESCC.

According to the World Health Organization, esophageal cancer is the sixth most common malignancy world wide.[1] The two predominant forms of esophageal cancer are squamous cell carcinoma and adenocarcinoma. Globally, squamous cell carcinoma accounts for more than 90% of esophageal cancer. Most esophageal squamous cell carcinoma (ESCC) is diagnosed at an advanced stage, and even superficial ESCC that appears to extend no further than the submucosa metastasizes to the lymph nodes in 50% of cases.[2] For localized ESCC, surgery is the primary therapeutic option. However, the prognosis is unsatisfactory, even in curatively resected patients where the 5-year survival rate is <50% after surgery.[3] Several prognostic markers, such as nodal status and tumor stage, are currently accepted for clinical use, and we have previously reported several ESCC-associated genes related to tumor progression.[4–6] However, these genes cannot completely identify which patients are at low or high risk for disease recurrence. Therefore, there is an urgent need for new prognostic markers and therapeutic targets for ESCC.

Better knowledge of changes in gene expression that occur during carcinogenesis may lead to improvements in diagnosis, treatment, and prevention of ESCC. Among the comprehensive methods used to analyze transcript expression levels, serial analysis of gene expression (SAGE) is a useful approach.[7,8] Previously, we performed SAGE analysis on one ESCC case, and identified several genes whose expression was upregulated or downregulated in ESCC. Of these genes, *ADAMTS16* is frequently overexpressed in ESCC.[9] However, expression of many genes remain unconfirmed, and their role in ESCC remain unclear.

In the present study, we reviewed a list of genes with upregulated expression in ESCC as identified by our SAGE analysis. We focused on the *NRD1* gene, which encodes the nardilysin protein, because nardilysin expression has not been investigated in ESCC. *NRD1* was initially cloned as a zinc metalloendopeptidase of the M16 family, which can selectively cleave the dibasic site *in vitro*.[10] In normal tissue, *NRD1* mRNA is expressed mainly in adult heart, skeletal muscle, and testis and at much lower levels in other tissues including thymus, prostate, ovary, small intestine, and leukocytes.[11] It has been reported that nardilysin enhances TNF-α shedding through activation of TNF-α converting enzyme (TACE) and a disintegrin and metalloprotease 10 (ADAM10).[12] In human breast cancer, it has been reported that nardilysin protein expression is correlated with tumor size, grade, and lymph node metastasis, but not prognosis.[13] Here, we examined the correlation between expression of nardilysin and prognosis in patients

with ESCC. We also revealed the biological function of nardilysin in esophageal cancer cell lines.

Materials and Methods

Tissue samples and cell lines. In total, 149 primary tumor samples were collected from patients diagnosed with ESCC. Patients were treated at the Hiroshima University Hospital. All patients underwent curative resection involving right transthoracic esophagectomy with extensive lymph node dissection. Reconstruction was performed with a gastric tube positioned in the posterior mediastinum. Only patients without preoperative radiotherapy or chemotherapy and without clinical evidence of distant metastasis were enrolled in the study. Operative mortality was defined as death within 30 days of patients leaving the hospital, and these patients were removed from the analysis. Postoperative follow-up was scheduled every 1, 2 or 3 months during the first 2 years after surgery and every 6 months thereafter unless more frequent follow-up was deemed necessary. Chest X-ray, chest computed tomography scan and serum chemistries were performed at every follow-up visit. Patients were followed by the patients' physician until their death or the date of the last documented contact. This study was approved by the Ethical Committee for Human Genome Research of Hiroshima University.

For quantitative reverse transcription-polymerase chain reaction (qRT-PCR), 40 ESCC tissue samples were used. Samples were frozen immediately in liquid nitrogen and stored at −80°C until use. Noncancerous samples of heart, lung, esophagus, stomach, small intestine, colon, liver, pancreas, kidney, bone marrow, peripheral leukocytes, spleen, skeletal muscle, brain, and spinal cord were purchased from Clontech (Palo Alto, CA, USA).

For Western blot analysis, four ESCC tissue samples and corresponding non-neoplastic mucosa samples were used. Samples were frozen immediately in liquid nitrogen and stored at −80°C until use.

For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from 109 patients who had undergone surgical excision for ESCC. Histological classification was based on the World Health Organization system. Tumor staging was performed according to the TNM stage grouping system.

Human esophageal cancer-derived cell lines, TE1, TE5, TE8, TE9, TE10, TE11 were purchased from RIKEN BioResource Center (Tsukuba, Japan). All cell lines were maintained in RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (FBS, Whittaker, Walkersville, MD, USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

RNA extraction and qRT-PCR. Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and 1 μg of total RNA was converted to cDNA with a First Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ, USA). Polymerase chain reaction was performed with a SYBR Green PCR Core Reagents Kit (Applied Biosystems, Foster City, CA, USA). *NRD1* primer sequences were 5′-TCT CAT AGG AGT CGC CTC TGC-3′ and 5′-CGA GTG GAG CCC TCA CAG AA-3′. Other primer sequences and additional PCR conditions are available upon request. Real-time detection of the emission intensity of SYBR green bound to double-stranded DNA was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as described previously.[14] *ACTB*-specific PCR products were amplified from the same RNA samples and served as internal controls.

Western blot analysis. For Western blot analysis, tissue samples or cells were lysed as described previously.[15] The lysates (40 μg) were solubilized in Laemmli sample buffer by boiling and then subjected to 8% SDS-polyacrylamide gel electrophoresis followed by electrotransfer onto a nitrocellulose filter. The filter was incubated with the primary antibody against nardilysin (mouse monoclonal; Abnova, Taipei, Taiwan). Peroxidase-conjugated anti-mouse IgG was used in the secondary reaction. Immunocomplexes were visualized with an ECL Western Blot Detection System (Amersham Biosciences). β-actin antibody (Sigma Chemical, St. Louis, MO, USA) was also used as a loading control.

Immunohistochemistry. One or two representative tumor blocks, including the tumor center, invading front, and tumor-associated non-neoplastic mucosa, was examined from each patient by immunohistochemistry. In cases of large, late-stage tumors, two different sections were examined to include representative areas of the tumor center as well as of the lateral and deep tumor invasive front. Immunohistochemical analysis was performed with a Dako Envision+ Mouse Peroxidase Detection System (Dako Cytomation, Carpinteria, CA, USA). Antigen retrieval was performed by microwave heating in citrate buffer (pH 6.0) for 30 min. Peroxidase activity was blocked with 3% H_2O_2-methanol for 10 min, and sections were incubated with normal goat serum (Dako Cytomation) for 20 min to block nonspecific antibody binding sites. Sections were incubated with a mouse monoclonal anti-nardilysin antibody (dilution 1:50) for 1 h at room temperature, followed by incubation with Envision+ anti-mouse peroxidase for 1 h. For color reaction, sections were incubated with the DAB Substrate-Chromogen Solution (Dako Cytomation) for 10 min. Sections were counterstained with 0.1% hematoxylin. Negative controls were created by omission of the primary antibody.

Expression of nardilysin was scored in all tumors as positive or negative. When more than 10% of tumor cells were stained, the immunostaining was considered positive for nardilysin. Using these definitions, two surgical pathologists (NU and NO), without knowledge of the clinical and pathologic parameters or the patients' outcomes, independently reviewed immunoreactivity in each specimen. Interobserver differences were resolved by consensus review at a double-headed microscope after independent review.

RNA interference. To knockdown the endogenous *NRD1*, RNA interference (RNAi) was performed. siRNA oligonucleotides for *NRD1* and a negative control were purchased from Invitrogen (Carlsbad, CA, USA). Three independent oligonucleotides were used for *NRD1* siRNA. The *NRD1* siRNA1 sequence was 5′-AUC AGU UGA GGC AUU AUC ACU ACC C-3′. The *NRD1* siRNA2 sequence was 5′-ACA GAC UGC AGC AAC AGU GAC UCU C-3′. The *NRD1* siRNA3 sequence was 5′-UAU UCU CUU AGA CUC CUG GUA CCU G-3′. Transfection was performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. Briefly, 60 pmol of siRNA and 10 μL of Lipofectamine RNAiMAX were mixed in 1 mL of RPMI medium (10 nmol/L final siRNA concentration). After 20 min of incubation, the mixture was added to the cells and these were plated on dishes for each assay. Forty-eight h after transfection, cells were analyzed for all experiments.

Cell growth, *in vitro* invasion, and wound healing assays. To examine cell growth, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed.[16] The cells

were seeded at a density of 2000 cells per well in 96-well plates. Cell growth was monitored after 1, 2, 4, and 8 days. Modified Boyden chamber assays were performed to examine invasiveness. Cells were plated at 10 000 cells per well in RPMI 1640 medium plus 1% serum in the upper chamber of a Transwell insert (8 μm pore diameter; Chemicon, Temecula, CA, USA) coated with Matrigel. Medium containing 10% serum was placed in the bottom chamber. After 1 and 2 days, cells in the upper chamber were removed by scraping, and the cells remaining on the lower surface of the insert were stained with CyQuant GR dye to assess the number of cells. To carry out the wound healing assay, the cells were plated onto collagen-coated coverslips. The monolayer cells were then scratched manually with a plastic pipette tip, and after being washed with PBS, the wounded monolayers of the cells were allowed to heal for 6–12 h in RPMI 1640 containing 10% FBS.

Statistical methods. Correlations between clinicopathologic parameters and nardilysin expression were analyzed by the χ^2 test. Kaplan–Meier survival curves were constructed for nardilysin-positive and nardilysin-negative patients. Survival rates were compared between nardilysin-positive and nardilysin-negative groups. Differences between survival curves were tested for statistical significance by the Log-rank test. Univariate and multivariate Cox regression was used to evaluate the associations between clinical covariates and survival. SPSS software was used for these analyses (SPSS Inc., Chicago, IL, USA). Hazard ratio (HR) and 95% confidence interval (CI) were estimated from Cox proportional hazard models. For all analyses, age was treated as a categorical variable (more than 65 years versus 65 years plus less than 65 years). For final multivariate Cox regression models, all variables were included that were moderately associated ($P < 0.10$) with survival by univariate analysis. A P-value of less than 0.05 was considered statistically significant.

Results

Upregulation of *NRD1* in ESCC. To identify novel biomarkers or therapeutic targets for ESCC, we reviewed a list of genes with upregulated expression in ESCC compared with normal esophagus, as identified by our SAGE analysis.[9] Among the 30 most upregulated genes in ESCC, we focused on the *NRD1* gene, which encodes the nardilysin protein, because expression of *NRD1* has not been investigated in ESCC, and an antibody against nardilysin is commercially available. We first performed qRT-PCR analysis of *NRD1* in 15 types of normal tissue samples, six esophageal cancer cell lines, and two ESCC tissue samples (Fig. 1a). Among the various normal tissue samples, abundant *NRD1* expression was found in normal pancreas and skeletal muscle, as reported previously.[11] Expression of *NRD1* in these normal tissue samples was greatest in skeletal muscle; however, expression of *NRD1* in ESCC tissue samples was even greater than in skeletal muscle.

Expression of nardilysin was also analyzed by Western blot in four ESCC tissue samples and their corresponding non-neoplastic mucosa samples. As shown in Figure 1b,c, upregulation of *NRD1* mRNA and nardilysin protein was detected in three ESCC tissue samples, and nardilysin protein expression and *NRD1* mRNA expression were well correlated. These results indicate that *NRD1* is upregulated in both mRNA and protein levels.

Next, we analyzed the expression of *NRD1* in additional 34 ESCC tissue samples and their corresponding non-neoplastic mucosa samples by qRT-PCR. When tumor/non-neoplastic

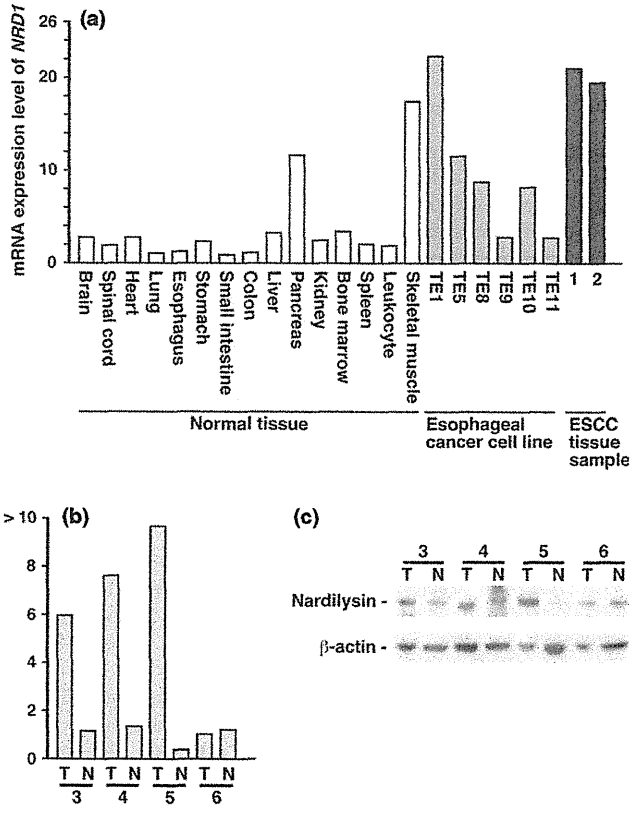

Fig. 1. Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis of *NRD1* mRNA and Western blot analysis of nardilysin protein in normal tissue samples, esophageal cancer cell lines, and esophageal squamous cell carcinoma (ESCC) tissue samples. (a) mRNA expression level of *NRD1* in 15 types of normal tissue samples, six esophageal cancer cell lines, and two ESCC tissue samples. The units are arbitrary, and *NRD1* mRNA expression was calculated by standardization of the expression in normal colon to 1.0. (b) mRNA expression level of *NRD1* in four ESCC tissue samples and their non-neoplastic mucosa samples. The units are arbitrary, and *NRD1* mRNA expression was calculated by standardization of the expression in non-neoplastic mucosa sample of case 3 to 1.0. (c) Nardilysin protein expression level in four ESCC tissue samples and their corresponding non-neoplastic mucosa samples analyzed in Figure 1b. T, Tumor; N, non-neoplastic mucosa.

esophagus mucosa ratios >2-fold higher were considered to represent upregulation, *NRD1* upregulation was observed in 19 (56%) of 34 ESCC cases.

Expression and distribution of nardilysin in ESCC tissue samples. Although we have demonstrated upregulation of nardilysin in ESCC tissue samples, the expression and distribution of nardilysin in ESCC remains unclear. To address this issue, immunohistochemical analysis of nardilysin was performed in ESCC tissue samples. We first tested the specificity of the anti-nardilysin antibody. In Western blot of protein extracts from the TE1 cell line, anti-nardilysin antibody detected a single band of approximately 133-kDa (data not shown).

We performed immunohistochemical analysis, first in normal skeletal muscle in which abundant *NRD1* mRNA expression was found, to serve as a positive control. Staining of nardilysin was observed in the normal skeletal muscle (Fig. 2a), consistent with our qRT-PCR results. Next, we performed immunohistochemical analysis in 109 ESCC tissue samples. In non-neoplastic esophageal mucosa, only weak or negative staining of nardilysin was observed in squamous epithelial

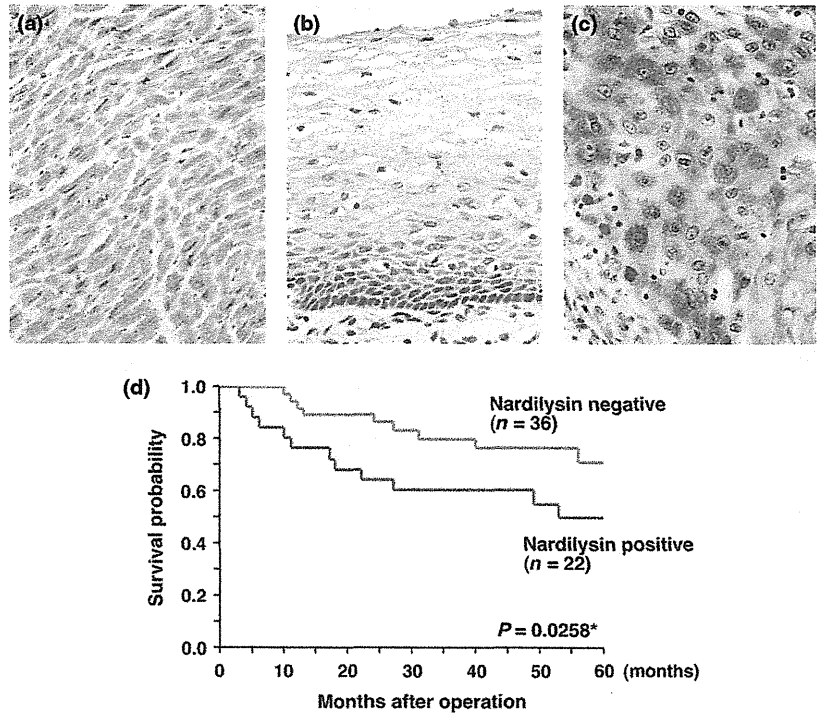

Fig. 2. Immunohistochemical analysis of nardilysin in normal tissue and esophageal squamous cell carcinoma (ESCC) tissue samples. (a) Immunohistochemical analysis of nardilysin in normal skeletal muscle (original magnification: ×200). (b) Immunohistochemical analysis of nardilysin in non-neoplastic esophageal mucosa (original magnification: ×400). (c) Immunohistochemical analysis of nardilysin in ESCC tissue (original magnification: ×400). (d) Prognostic value of nardilysin staining. In patients with ESCC who did not receive adjuvant or neoadjuvant therapy ($n = 58$), patients with onardilysin-positive ESCC had a worse survival rate than patients with nardilysin-negative ESCC. *Log-rank test.

and stromal cells (Fig. 2b). In contrast, ESCC tissue showed stronger or more extensive staining than corresponding non-neoplastic esophageal mucosa (Fig. 2c). Staining of nardilysin was observed in the cytoplasm. Some ESCC cases showed heterogeneity of immunostaining of nardilysin, but a tendency for upregulation of nardilysin at the invasive front was not observed. Because some ESCC tissue samples showed heterogeneity of nardilysin immunostaining, we considered nardilysin staining to be positive when more than 10% of tumor cells were stained. In total, nardilysin-positive ESCC cases were found in 43 (39%) of 109 cases. We analyzed the relationship between nardilysin expression and clinicopathologic characteristics. Nardilysin-positive ESCC cases were more advanced in terms of T classification ($P = 0.0007$, χ^2 test), N classification ($P = 0.0164$, χ^2 test), and tumor stage ($P < 0.0001$, χ^2 test) than nardilysin-negative ESCC cases (Table 1). In addition, nardilysin-positive ESCC cases were more frequently found in moderately/poorly differentiated ESCC than in well differentiated ESCC cases ($P = 0.0200$, χ^2 test). Expression of nardilysin was not associated with age or sex.

Relationship between expression of nardilysin in ESCC and prognosis. The association between nardilysin expression and prognosis was investigated by Kaplan–Meier analysis of patients with ESCC who did not receive adjuvant or neoadjuvant therapy ($n = 58$). Nardilysin expression was significantly associated with poorer prognosis ($P = 0.0258$, Log-rank test, Fig. 2d). Univariate and multivariate Cox proportional hazards analysis was used to further evaluate the association between nardilysin expression and survival in patients with ESCC who did not receive adjuvant or neoadjuvant therapy ($n = 58$, Table 2). In univariate analysis, nardilysin expression (hazard ratio [HR] 2.574; 95% confidence interval [CI] 1.087–6.313; $P = 0.0315$) and tumor stage (HR 8.919; 95% CI 3.585–25.215; $P < 0.0001$) were associated with survival. We also performed a multivariate model, which included nardilysin expression and tumor stage. Nardilysin expression was an independent prognostic predictor for survival in patients with ESCC (HR 2.476; 95% CI 1.039–6.115; $P = 0.0407$).

Table 1. Relationship between nardilysin expression and clinicopathologic characteristics in esophageal squamous cell carcinoma

	Nardilysin expression		*P*-value†
	Positive	Negative	
Age			
≤65	16 (33%)	33	0.1880
>65	27 (45%)	33	
Sex			
Male	37 (39%)	59	0.6008
Female	6 (46%)	7	
T classification			
T1	12 (29%)	40	0.0007
T2/3/4	31 (54%)	26	
N classification			
N0	16 (29%)	40	0.0164
N1/2/3/4	27 (51%)	26	
Stage			
Stage 0/I	6 (15%)	33	< 0.0001
Stage II/III/IV	37 (53%)	33	
Histological classification			
Well	11 (26%)	31	0.0200
Moderately/poorly	32 (48%)	35	

†χ^2 test.

Effect of *NRD1* inhibition on cell growth, invasive activity and cell motility in esophageal cancer cells. We showed that high levels of *NRD1* mRNA expression were correlated with T classification, N classification and tumor stage in ESCC tissues. Furthermore, nardilysin protein expression was correlated with patients' prognosis. Therefore, we studied the biological significance of *NRD1* using esophageal cancer cell lines.

Table 2. Univariate and multivariate Cox regression analysis of nardilysin expression and survival in esophageal squamous cell carcinoma

Characteristic	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Nardilysin expression				
Negative	1 (Ref.)	0.0315	1 (Ref.)	0.0407
Positive	2.574 (1.087–6.313)		2.476 (1.039–6.115)	
Tumor stage				
0/I/II	1 (Ref.)	<0.0001	1 (Ref.)	<0.0001
III/IV	8.919 (3.585–25.215)		8.895 (3.547–25.306)	
Age				
≤65	1 (Ref.)	0.4506		
>65	1.391 (0.586–3.343)			
Sex				
Female	1 (Ref.)	0.8663		
Male	1.109 (0.375–4.739)			
Histological classification				
Well	1 (Ref.)	0.9035		
Moderately/ Poorly	1.064 (0.416–3.259)			

CI, confidence interval; HR, hazard ratio.

Western blot analysis showed that all six esophageal cancer cell lines expressed nardilysin at various levels (Fig. 3a). *NRD1* mRNA expression and nardilysin protein expression were well correlated. The highest nardilysin expression was detected in TE1 cells, and the other five remaining cell lines had moderate or low nardilysin expression. Next, we examined the transition of nardilysin expression by Western blot analysis of protein extracts of TE1 and TE5 cell lines transfected with *NRD1* specific siRNAs because the highest nardilysin expression was detected in TE1 cells, and moderate nardilysin expression was detected in TE5 cells. Three different siRNAs (siRNA1, 2, and 3) were transfected into TE1 and TE5 (Fig. 3b). The expression of nardilysin protein in TE1 was most suppressed by treatment with siRNA1. Similar results were observed in TE5 cells. Thus, to knockdown the endogenous *NRD1*, we used siRNA1 in the following experiments.

To investigate the possible antiproliferative effects of *NRD1* knockdown, we performed an MTT assay 8 days after siRNA transfection. The viability of *NRD1* siRNA1-transfected TE1 cells was not significantly different from that of negative control siRNA-transfected TE1 cells (data not shown). We performed the same assay in TE5 cells, and similar results were obtained. Next, to determine the possible role of *NRD1* in the invasiveness of esophageal cancer cells, we used a transwell invasion assay (Fig. 3c). We performed three different experiments and calculated mean and standard error (SE). On day 2, although there was no difference in cell viability between *NRD1* knockdown TE1 cells and negative control siRNA-transfected TE1 cells, the invasiveness of *NRD1* knockdown TE1 cells was less than that of the negative control siRNA-transfected TE1 cells. Similar results were obtained in TE5 cells. The wound healing assay was also performed in TE1 cells (Fig. 3d). The migration activity of *NRD1* knockdown TE1 cells was significantly lower than that of negative control siRNA-transfected TE1 cells. Similar results were obtained in TE5 cells. These results indicate that *NRD1* promotes invasion activity in esophageal cancer cells.

***NRD1* knockdown inhibits MMP2 expression.** It is important to establish why *NRD1* knockdown inhibits cell invasion activity. It is well known that the matrix metalloproteinase (MMP) family is involved in cell invasion activity. It has been reported that *MMP1*, *MMP2*, *MMP3*, *MMP9*, and *MMP10* are involved in esophageal cancer.[17] Therefore, expression of these MMPs was measured in *NRD1* knockdown cells. We performed three different experiments and calculated mean and SE. As shown in Figure 3e, expression of *MMP2* and *MMP3* mRNA was significantly lower in *NRD1* knockdown TE5 cells than in negative control siRNA-transfected TE5 cells. Expression of *MMP2* and *MMP3* mRNA did not significantly differ between *NRD1* knockdown TE1 cells and negative control siRNA-transfected TE1 cells. Expression of *MMP1*, *MMP9*, and *MMP10* mRNA was not significantly different between *NRD1* knockdown cells and negative control siRNA-transfected cells.

Discussion

The long-term survival of patients with ESCC remains poor due to the high incidence of lymph node metastasis and early recurrence after curative surgical resection. In the present study, to identify novel prognostic markers or therapeutic targets for ESCC, we reviewed a list of genes whose expression was upregulated in ESCC compared with normal esophagus, as identified by our SAGE analysis. We found that *NRD1* was upregulated in ESCC, and confirmed by immunohistochemistry that nardilysin protein was expressed in ESCC cells. Furthermore, univariate and multivariate analyses revealed that nardilysin expression is an independent prognostic classifier of patients with ESCC. These results indicate that immunohistochemical analysis of nardilysin is a clinically useful method for prediction of ESCC patient survival.

In the present study, nardilysin protein expression was correlated with advanced T classification, N classification, and tumor stage. Kaplan–Meier analysis revealed that nardilysin expression was significantly associated with poorer prognosis in patients with ESCC who did not receive adjuvant or neoadjuvant therapy. Furthermore, the invasiveness of *NRD1* knockdown cells was 60% less than that of the negative control siRNA-transfected cells. These results indicate that nardilysin was involved in tumor progression. Although expression of *NRD1* mRNA was found in normal pancreas and skeletal muscle, and was not specific to ESCC, expression levels of *NRD1* mRNA in ESCC were higher than skeletal muscle or pancreas. Thus, *NRD1* could be a good therapeutic target with less adverse effects for ESCC.

Although we showed that knockdown of *NRD1* inhibits invasion activity in esophageal cancer cell lines, the underlying mechanisms remain unclear. We found that expression of *MMP2* and *MMP3* mRNA could be inhibited by *NRD1* knockdown. Although *NRD1* knockdown inhibited cell invasion activity in both TE1 and TE5 cells, inhibition of *MMP2* and *MMP3* mRNA expression was only observed in TE5 cells, and not in TE1 cells. The migration activities of *NRD1*-knockdown TE1 and TE5 cells analyzed by wound healing assay were significantly lower than those of negative control siRNA-transfected TE1 and TE5 cells. Therefore, inhibition of invasion

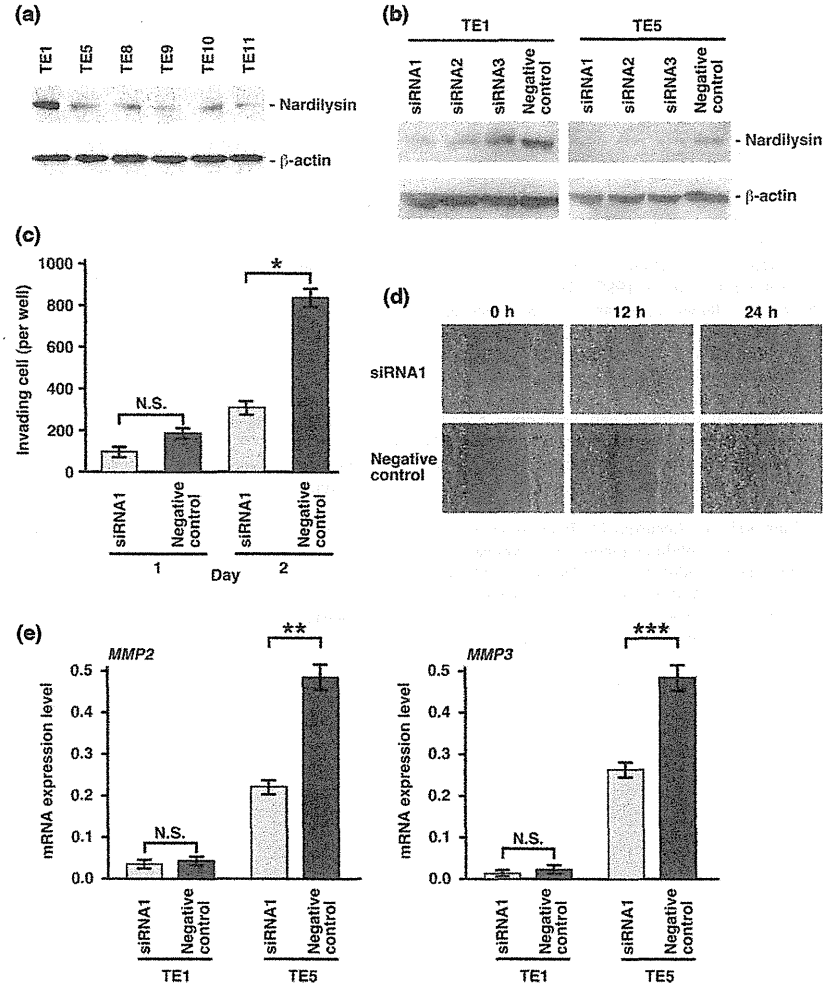

Fig. 3. Functional analysis of nardilysin in esophageal cancer cell lines. (a) Western blot analysis of nardilysin in six esophageal cancer cell lines. (b) Western blot analysis of nardilysin in cell lysates from TE1 and TE5 cells transfected with *NRD1* siRNA (siRNA1–3) and negative control siRNA. (c) Effect of *NRD1* knockdown on cell invasion of TE1. TE1 cells transfected with *NRD1* siRNA1 and negative control siRNA were incubated in Boyden chambers. After 1 and 2 days, invading cells were counted. Bars and error bars indicate mean and SE of three different experiments. (d) Wound healing assay in *NRD1* knockdown TE1 cells and negative control siRNA-transfected TE1 cells. TE1 cells transfected with *NRD1* siRNA1 or negative control siRNA were wounded. (e) Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis of *MMP2* and *MMP3* mRNA in TE1 and TE5 cells transfected with *NRD1* siRNA1 or negative control siRNA. Bars and error bars represent mean and standard error (SE) of three different experiments. N.S., not significant. $*P = 0.0244$; $**P = 0.0200$; $***P < 0.0200$.

activity by *NRD1* knockdown is likely to be due to inhibition of migration activity rather than inhibition of *MMP2* or *MMP3* expression. It has been reported that heparin-binding epidermal growth factor-like growth factor (HB-EGF) binds to nardilysin, and transient expression of nardilysin in HeLa cells increased migration in response to HB-EGF.[18] Because nardilysin-induced migration occurs via the EGF receptor (EGFR),[18] it is possible that inhibition of EGFR signaling is involved in reduction of migration activity by *NRD1* knockdown. In support of this, high expression of EGFR has been reported in TE1 and TE5 cells.[19,20] It has also been reported that nardilysin is one of the mutant p53-specific binding partners, and that interaction of mutant p53 with nardilysin enhances invasion activity.[21] Because our previous study revealed that p53 mutation status is not associated with prognosis of patients with ESCC,[4] expression of nardilysin may affect prognosis in patients with ESCC showing p53 mutation.

In summary, we have shown that nardilysin expression is an independent prognostic classifier in patients with ESCC. It is possible that immunohistochemical analysis of nardilysin may help identify patients who would benefit from adjuvant chemotherapy. Further analysis will help to elucidate the biological function and prognostic value of nardilysin in ESCC.

Acknowledgments

We thank Mr Shinichi Norimura for his excellent technical assistance and advice. This work was carried out with the kind cooperation of the Research Center for Molecular Medicine of the Faculty of Medicine of Hiroshima University. We also thank the Analysis Center of Life Science of Hiroshima University for the use of their facilities. This work was supported in part by grants-in-aid for Cancer Research from the Ministry of Education, Culture, Science, Sports and Technology of Japan and in part by a grant-in-aid for the Third Comprehensive 10-year Strategy for Cancer Control from the Ministry of Health, Labor and Welfare of Japan, and by The National Institute of Biomedical Innovation (Program for Promotion of Fundamental Studies in Health Sciences) and by National Cancer Center Research and Development Fund.

Disclosure Statement

The authors have no conflict of interest.

References

1 Stoner GD, Gupta A. Etiology and chemoprevention of esophageal squamous cell carcinoma. *Carcinogenesis* 2001; **22**: 1737–46.

2 Goseki N, Koike M, Yoshida M. Histopathologic characteristics of early stage esophageal carcinoma. A comparative study with gastric carcinoma. *Cancer* 1992; **69**: 1088–93.

3 Courrech Staal EF, van Coevorden F, Cats A *et al.* Outcome of low-volume surgery for esophageal cancer in a high-volume referral center. *Ann Surg Oncol* 2009; **16**: 3219–26.
4 Takeno S, Noguchi T, Kikuchi R, Uchida Y, Yokoyama S, Müller W. Prognostic value of cyclin B1 in patients with esophageal squamous cell carcinoma. *Cancer* 2002; **94**: 2874–81.
5 Noguchi T, Oue N, Wada S *et al.* h-Prune is an independent prognostic marker for survival in esophageal squamous cell carcinoma. *Ann Surg Oncol* 2009; **16**: 1390–6.
6 Oue N, Noguchi T, Anami K *et al.* Cytokeratin 7 is a predictive marker for survival in patients with esophageal squamous cell carcinoma. *Ann Surg Oncol* 2012; **19**: 1902–10.
7 Velculescu VE, Zhang L, Vogelstein B, Kinzler KW. Serial analysis of gene expression. *Science* 1995; **270**: 484–7.
8 Oue N, Hamai Y, Mitani Y *et al.* Gene expression profile of gastric carcinoma: identification of genes and tags potentially involved in invasion, metastasis, and carcinogenesis by serial analysis of gene expression. *Cancer Res* 2004; **64**: 2397–405.
9 Sakamoto N, Oue N, Noguchi T *et al.* Serial analysis of gene expression of esophageal squamous cell carcinoma: ADAMTS16 is upregulated in esophageal squamous cell carcinoma. *Cancer Sci* 2010; **101**: 1038–44.
10 Pierotti AR, Prat A, Chesneau V *et al.* N-arginine dibasic convertase, a metalloendopeptidase as a prototype of a class of processing enzymes. *Proc Natl Acad Sci USA* 1994; **91**: 6078–82.
11 Fumagalli P, Accarino M, Egeo A *et al.* Human NRD convertase: a highly conserved metalloendopeptidase expressed at specific sites during development and in adult tissues. *Genomics* 1998; **47**: 238–45.
12 Hiraoka Y, Yoshida K, Ohno M, Matsuoka T, Kita T, Nishi E. Ectodomain shedding of TNF-alpha is enhanced by nardilysin via activation of ADAM proteases. *Biochem Biophys Res Commun* 2008; **370**: 154–8.
13 Choong LY, Lim SK, Chen Y *et al.* Elevated NRD1 metalloprotease expression plays a role in breast cancer growth and proliferation. *Genes Chromosom Cancer* 2011; **50**: 837–47.
14 Kondo T, Oue N, Yoshida K *et al.* Expression of POT1 is associated with tumor stage and telomere length in gastric carcinoma. *Cancer Res* 2004; **64**: 523–9.
15 Yasui W, Ayhan A, Kitadai Y *et al.* Increased expression of p34cdc2 and its kinase activity in human gastric and colonic carcinomas. *Int J Cancer* 1993; **53**: 36–41.
16 Alley MC, Scudiero DA, Monks A *et al.* Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res* 1988; **48**: 589–601.
17 Groblewska M, Siewko M, Mroczko B, Szmitkowski M. The role of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) in the development of esophageal cancer. *Folia Histochem Cytobiol* 2012; **50**: 12–19.
18 Nishi E, Prat A, Hospital V, Elenius K, Klagsbrun M. N-arginine dibasic convertase is a specific receptor for heparin-binding EGF-like growth factor that mediates cell migration. *EMBO J* 2001; **20**: 3342–50.
19 Suwa T, Ueda M, Jinno H *et al.* Epidermal growth factor receptor-dependent cytotoxic effect of anti-EGFR antibody-ribonuclease conjugate on human cancer cells. *Anticancer Res* 1999; **19**: 4161–5.
20 Ichiba M, Miyazaki Y, Kitamura S, Kiyohara T, Shinomura Y, Matsuzawa Y. Epidermal growth factor inhibits the growth of TE8 esophageal cancer cells through the activation of STAT1. *J Gastroenterol* 2002; **37**: 497–503.
21 Coffill CR, Muller PA, Oh HK *et al.* Mutant p53 interactome identifies nardilysin as a p53R273H-specific binding partner that promotes invasion. *EMBO Rep* 2012; **13**: 638–44.

Cancer Science
Japanese Cancer Association JCA
Open Access

MicroRNA-143 regulates collagen type III expression in stromal fibroblasts of scirrhous type gastric cancer

Yutaka Naito,[1] Naoya Sakamoto,[1] Naohide Oue,[1] Masakazu Yashiro,[2] Kazuhiro Sentani,[1] Kazuyoshi Yanagihara,[3] Kosei Hirakawa[2] and Wataru Yasui[1]

[1]Department of Molecular Pathology, Institute of Biomedical and Health Sciences, Hiroshima University, Hiroshima; [2]Department of Surgical Oncology, Graduate School of Medicine, Osaka City University, Osaka; [3]Division of Translational Research, Exploratory Oncology and Clinical Trial Center, National Cancer Center, Chiba, Japan

Key words

Collagen type III, fibroblast, gastric cancer, microRNA-143, transforming growth factor-β

Correspondence

Wataru Yasui, Department of Molecular Pathology, Institute of Biomedical and Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan.
Tel: +81-82-257-5145; Fax: +81-82-257-5149;
E-mail: wyasui@hiroshima-u.ac.jp

Funding information

Ministry of Education, Culture, Science, Sports, and Technology of Japan. Ministry of Health, Labour and Welfare of Japan. The National Institute of Biomedical Innovation. Japan Society for the Promotion of Science. National Cancer Center Research and Development Fund.

Received July 16, 2013; Revised November 24, 2013; Accepted November 26, 2013

Cancer Sci **105** (2014) 228–235

doi: 10.1111/cas.12329

Gastric cancer (GC) is one of the most common malignancies worldwide. In particular, scirrhous type GC is highly metastatic and is characterized clinically by rapid disease progression and poor prognosis. MicroRNAs (miRNAs) play crucial roles in cancer development and progression. In the present study, we identified several miRNAs that are expressed at higher levels in scirrhous type GC than in non-scirrhous type GC by miRNA microarray analysis. Among these, microRNA-143 (miR-143) expression was higher in scirrhous type GC than in non-scirrhous types of GC. *In situ* hybridization and quantitative RT-PCR analysis showed that miR-143 is expressed by stromal fibroblasts but not by cancer cells. In stromal cells, miR-143 enhanced collagen type III expression in normal gastric fibroblasts and cancer-associated fibroblasts through activation of transforming growth factor-β)/SMAD signaling. Furthermore, high miR-143 expression in GC was associated with worse cancer-specific mortality ($P = 0.0141$). Multivariate analysis revealed that miR-143 was an independent prognostic factor. Treatment of GC cell lines with 5-aza-2′-deoxycytidine restored the expression of miR-143, and precursor miR-143 caused the inhibition of cancer cell invasion. These data suggest that miR-143 regulates fibrosis of scirrhous type GC through induction of collagen expression in stromal fibroblasts and that miR-143 expression serves as a prognostic marker of GC.

Gastric cancer (GC) is one of the most common malignancies worldwide. Although improved diagnosis and treatment have resulted in good long-term survival for patients with early GC, outcomes for those with advanced GC remain poor.[1] Gastric cancer can be subdivided into two major classifications, intestinal-type GC and diffuse-type GC.[2] Scirrhous type GC, composed mainly of diffuse-type GC cells, forms a Borrmann type 4 lesion and is characterized by highly metastatic potential and rapid proliferation.[3–5] Histologically, scirrhous type GC shows diffuse infiltration into the gastric wall with extreme stromal fibrosis. Transforming growth factor-β (TGF-β), produced by cancer cells, has been reported to activate stromal fibroblasts to stimulate collagen synthesis in scirrhous type GC.[6,7] Increasing matrix rigidity may lead to the activation of proliferation, and interstitial pressure by fibrosis in the cancer stroma may interfere with drug delivery to cancer cells.[8–11] Reflecting such characteristics, scirrhous type GC carries an extremely poor patient prognosis in comparison with other types of GC. Therefore, better knowledge of the pathological and biological basis of scirrhous type GC is necessary to improve diagnosis and treatment.

MicroRNAs (miRNAs) are small non-coding RNAs of 19–25 nucleotides in length that play important regulatory roles in posttranscriptional repression.[12,13] Through inhibition of target gene translation, miRNAs regulate many cellular processes including development, differentiation, stress response, apoptosis, and proliferation. Aberrant miRNA expression is found in a range of cancers, suggesting novel roles as oncogenes or tumor-suppressor genes.[14] Several reports indicated significant correlations between the histological classification of cancers and miRNA expression patterns.[14,15] We have previously reported that the two histological types of GC, intestinal-type and diffuse-type, show different miRNA signatures.[16] However, there is only one report focusing on scirrhous type GC, which found that miR-516a-3p participated in inhibition of peritoneal metastasis.[17]

In this study, we aimed to identify novel miRNAs in scirrhous type GC by comparing miRNA expression profiles of GC tissues and found that miR-143 expression levels in scirrhous type GC were higher than in other types of GC. It has been shown that miR-143 expression is induced by TGF-β signaling, and it regulates vascular smooth muscle cell differentiation.[18] Moreover, several lines of evidence support the