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

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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.

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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* *DCC* *SPN*	M, 6[a]; U, 5[b]
MSI (MMR gene)	MSI/BAT	M, 4[a]; U, 8[b]
Cell proliferation	Polyamine	
Growth factor/ cytokine and those receptor	VEGF IL6 IGF cMet EGFR	M, 2[a]; U, 6[b]
Apoptosis signal pathway	TRAIL	
Cell invasion and adhesion	MRP-1 uPA Matrilysin S100A4 Angiomodulin CD44 Laminin β6-integrin α3-integrin	M, 1[a]; U, 3[b]
Angiogenesis	CD105	
Others	Vascular density Galectin CD95 Telomerase	M, 3[a]; U, 2[b]

[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

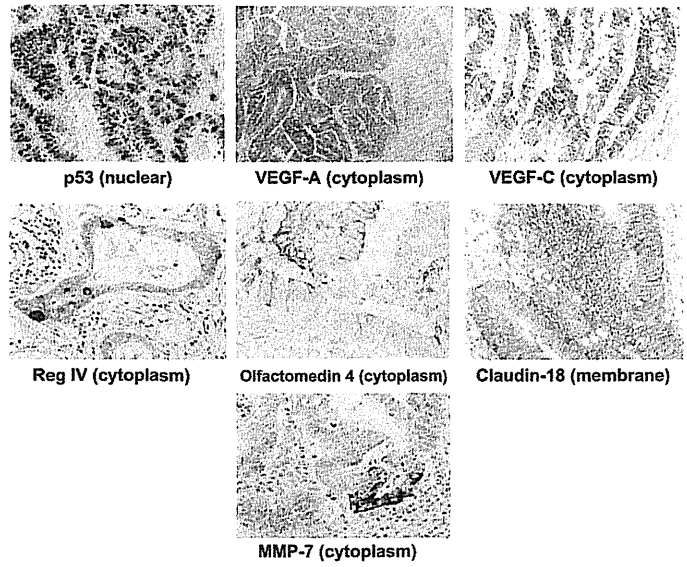

Figure 1. Immunohistochemical determination of p53, VEGF-A, VEGF-C, Reg IV, olfactomedin 4, Claudin-18 and MMP-7. p53 was found in the nuclei of cancer cells. Claudin-18 was observed at cell-cell boundaries of cancer cells. Other molecules were found in the cytoplasm of cancer cells.

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

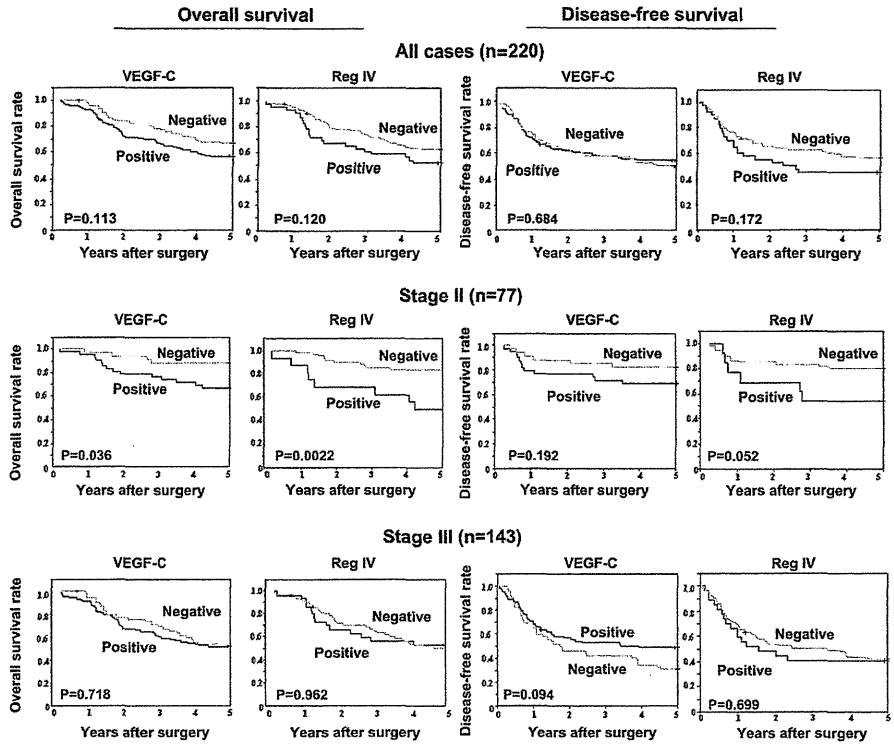

Figure 2. The overall (OS) and disease-free survival (DFS) curves of 220 colorectal cancer cases. Colorectal cancer with VEGF-C-positive expression showed a significantly worse OS time (P=0.036), and cases with Reg IV expression tend to have the worst OS time (P=0.0022).

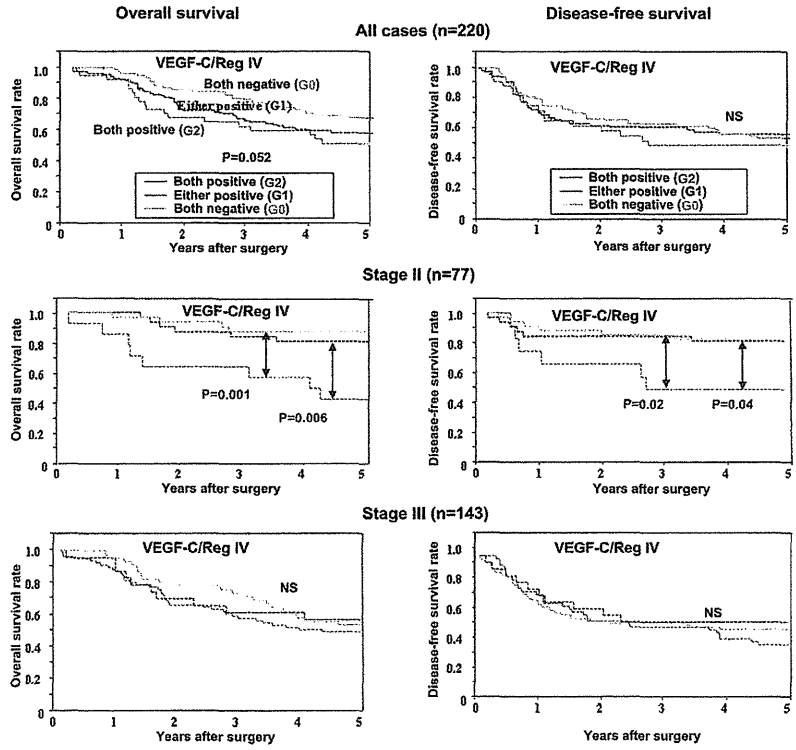

Figure 3. Survival of patients with colorectal cancer based on VEGF-C and Reg IV expression. In patients at stage II, the overall survival (OS) of the combination of VEGF-C and Reg IV positive group (G2) was significantly poorer than that of the combination of either positive (G1) and both negative group (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.

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

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Esophageal cancer, invasion, nardilysin, NRD1, prognosis

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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

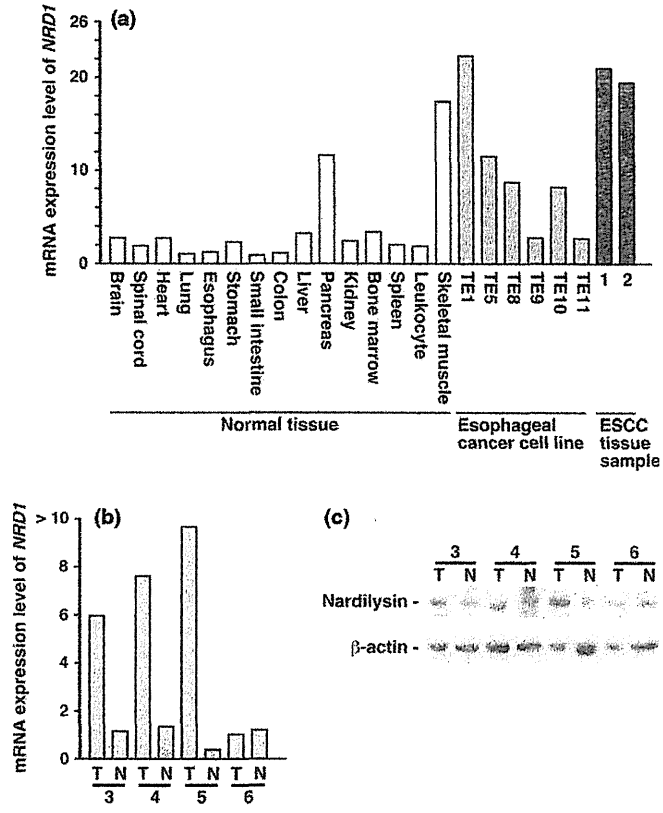
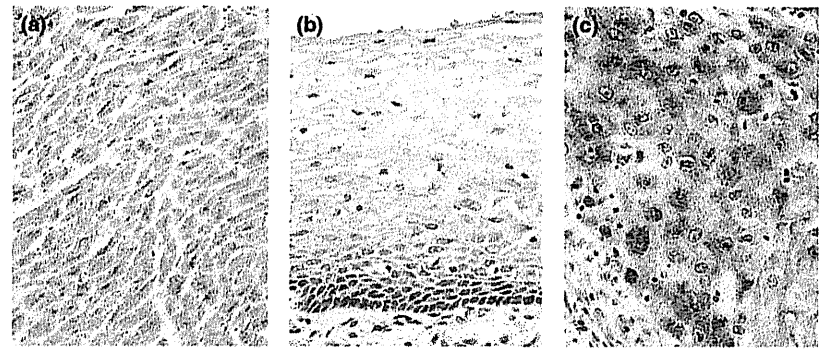


Fig. 1. Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis of *NRD1* mRNA and Western blot analysis of nardilysin protein in normal tissue samples, esophageal cancer cell lines, and esophageal squamous cell carcinoma (ESCC) tissue samples. (a) mRNA expression level of *NRD1* 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



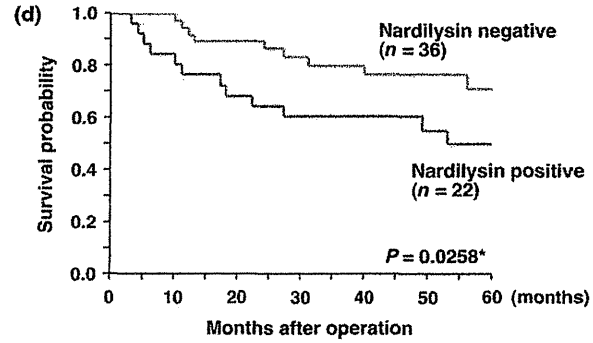


Fig. 2. Immunohistochemical analysis of nardilysin in normal tissue and esophageal squamous cell carcinoma (ESCC) tissue samples. (a) Immunohistochemical analysis of nardilysin in normal skeletal muscle (original magnification: ×200). (b) Immunohistochemical analysis of nardilysin in non-neoplastic esophageal 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

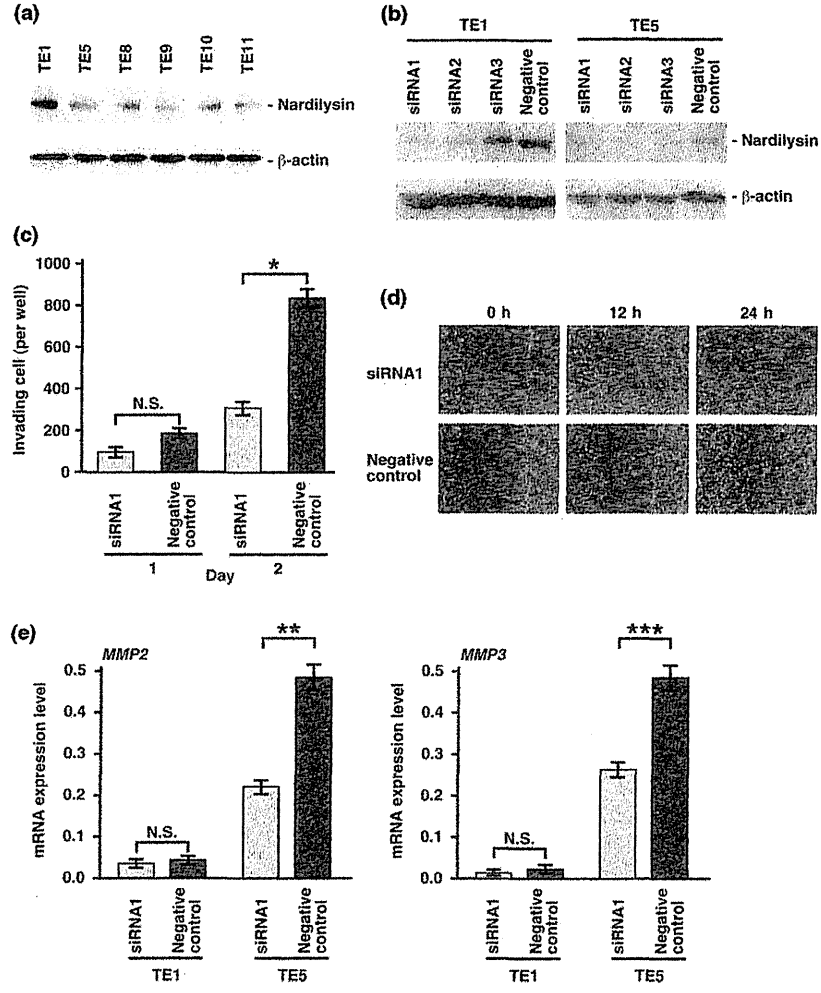


Fig. 3. Functional analysis of nardilysin in esophageal cancer cell lines. (a) Western blot analysis of nardilysin in six esophageal cancer cell lines. (b) Western blot analysis of nardilysin in cell lysates from TE1 and TE5 cells transfected with *NRD1* siRNA (siRNA1–3) and negative control siRNA. (c) 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.

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Disclosure Statement

The authors have no conflict of interest.

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

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

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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

Table 1. Summary of significantly increased miRNAs in scirrhous type GC, compared with non-scirrhous type GC

miRBase ID	miRBase Accession No.	Intensity ave.		Fold change	*P* value
		Scirrhous	Non-scirrhous		
hsa-miR-143	MIMAT0000435	21598.00	4937.81	4.37	0.0060
hsa-miR-145	MIMAT0000437	19051.80	3668.44	5.19	0.0049
hsa-miR-125b	MIMAT0000423	2076.80	556.50	3.73	0.0031
hsa-miR-99a	MIMAT0000097	908.00	231.25	3.93	0.0036
hsa-miR-100	MIMAT0000098	792.60	211.69	3.74	0.0019
hsa-miR-17-3p	MIMAT0000071	156.00	41.44	3.76	0.0498

importance of miR-143 in proliferation, invasion, and metastasis of various malignancies.[19–22] By *in situ* hybridization, miR-143 was localized in stromal fibroblasts but not in GC cells of scirrhous type GC tissue. Here, we investigated the function of miR-143 in stromal cells in scirrhous type GC, particularly in collagen type III synthesis by stromal fibroblasts. The expression of collagen type III was positively regulated by miR-143 through the TGF-β/SMAD signaling pathway. We also examined the correlation between miR-143 expression and patient prognosis using clinicopathological characteristics.

Materials and Methods

MicroRNA microarray hybridization. Total RNA was isolated from frozen tissue using Isogene (Nippon Gene, Tokyo, Japan). Short-strand RNA was purified from total RNA with RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA, USA). The oligonucleotide array we used contained Genopal-MICH07 DNA chips (Mitsubishi Rayon, Tokyo, Japan) comprising 188 oligonucleotide DNA probes. Details are described in Data S1.

Tissue samples. In all, 138 primary gastric tumors and 30 corresponding non-neoplastic mucosa were collected from patients diagnosed as having GC. Details are described in Data S1.

Cell culture. Nine cell lines derived from human GC and four human normal gastric fibroblasts (NFs), NF-33, -34, -35, and -38, and four cancer-associated fibroblasts (CaFs), CaF-33, 34, 35, and 38, were used. These cell lines were maintained as described previously.[23,24] Additional information on the NFs and CaFs is provided in Table S1, and details are described in Data S1.

Quantitative RT-PCR and western blots. Quantification of levels of collagen type III mRNA, α-smooth muscle actin (α-SMA) mRNA, β-actin mRNA, TGF-β variants mRNA, miR-143, and U6B was carried out using real-time fluorescence detection. For Western blot analysis, cells were lysed as described previously.[25] Details are described in Data S1.

***In situ* hybridization for miR-143 in combination with immunofluorescence staining and immunostaining of collagen.** *In situ*

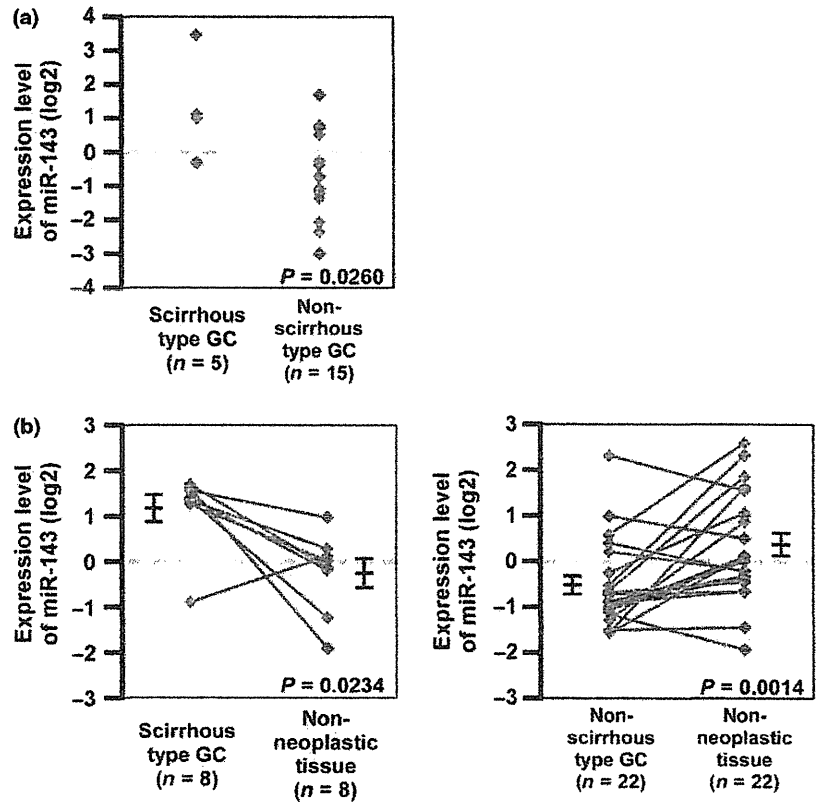

Fig. 1. MicroRNA-143 (miR-143) expression in gastric cancer (GC) and non-neoplastic tissue. (a) Expression levels of miR-143 in GC tissue samples ($n = 20$) were measured by quantitative RT-PCR analysis. (b) MicroRNA-143 expression levels in 30 formalin-fixed paraffin-embedded GC tissue samples and non-neoplastic tissue samples as determined by quantitative RT-PCR were compared. Statistical differences were evaluated using the Wilcoxon matched pair test. Bars and error bars indicated median and standard error.

hybridization was carried out as described by Nuovo *et al.*[26] with minor adjustments. A Dako EnVision+ Mouse Peroxidase Detection Kit (Dako, Carpinteria, CA, USA) was used for immunohistochemical analysis. Details are described in Data S1.

Cell transfection and TGF-β1 treatment. Transfection of cells was carried out with Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Fibroblasts were incubated in DMEM containing 10 ng/mL TGF-β1 (R&D Systems, Minneapolis, MO, USA). Details are described in Data S1.

Cell growth and *in vitro* invasion assay. The cells were seeded at a density of 2000 cells per well in 96-well plates. Cell growth was monitored after 1, 2, and 4 days by MTT assay.[27] Modified Boyden chamber assays were carried out to examine invasiveness as described previously.[28]

Immunofluorescence staining for cell lines. For cell staining, the cells were incubated with anti-collagen type III antibody (Daiichi Fine Chemical, Toyama, Japan), followed by incubations with Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA). Details are described in Data S1.

Coculture of CaF with scirrhous type GC cell line and BrdU incorporation assay. HSC-44PE, scirrhous type GC cell line, was cocultured with CaF-38, and proliferation activity was assessed by percentage of BrdU/CAM5.2-positive cells. Details are described in Data S1.

Statistical analysis. The Mann–Whitney *U*-test was used to calculate the significance of differences between two samples. Statistical differences between miRNA expression levels in GC samples and non-neoplastic mucosa samples were evaluated using the Wilcoxon matched pair test. The correlation between expression levels of miR-143 and clinicopathological parameters was analyzed with Fisher's exact test. A log–rank test and Kaplan–Meier plots were constructed for the miR-143 high and low groups, based on one-third of the miR-143 expression level. Univariate and multivariate analysis of factors influencing survival were carried out using the Cox proportional hazards model. Parameters for multivariate analysis were selected by the stepwise method. A *P*-value of less than 0.05 was considered statistically significant.

Results

Expression of miR-143 is greater in scirrhous type GC than in non-scirrhous type GC. To identify miRNAs with altered expression levels among different histological types of GC, expression levels of 188 individual miRNAs were compared between five scirrhous type GCs and 15 non-scirrhous type GCs in miRNA microarray profiling. Expression levels of six miRNAs were significantly higher in scirrhous type GC than in non-scirrhous type GC (Table 1). Among these, miR-143 was expressed at the highest level in scirrhous type GC. To confirm the microarray data, we carried out quantitative RT-PCR (qRT-PCR) in 20 frozen GC tissue samples. As shown in Figure 1(a), miR-143 expression in scirrhous type GC was significantly higher than that in non-scirrhous type GC.

MicroRNA-143 can act as a tumor suppressor gene, and its expression is decreased in tumor tissues relative to normal tissues.[19,20,29] To assess miR-143 expression between cancer tissue and non-neoplastic tissue from the same patients, we carried out qRT-PCR analyses of miR-143 using 30 formalin-fixed paraffin-embedded GC tissue samples and corresponding non-neoplastic gastric mucosa samples. Although miR-143 expression levels were significantly lower in non-scirrhous type GC tissues than in non-neoplastic gastric mucosa, the expression levels in scirrhous type GC were significantly higher than those in the corresponding non-neoplastic mucosa (Fig. 1b). These data suggest that miR-143 expression is downregulated in GCs but is sustained or increased in scirrhous type GC.

Analysis of miR-143 localization in scirrhous type GC. To elucidate why scirrhous-type GC possesses high miR-143 expression, we first investigated the localization of miR-143 expression in scirrhous type GC tissue by *in situ* hybridization of miR-143 in combination with immunostaining using markers for epithelial cells (CAM5.2), stromal fibroblasts (vimentin), and CaFs (α-SMA; Fig. 2a–c, Fig. S1).[30] Cancer fibroblasts, also termed myofibroblasts or activated fibroblasts, are well known as a major component of cancer stroma and play an important role in the regulation of cancer cell proliferation and metastasis.[5,30] In cancerous regions, double staining revealed that expression of miR-143 was observed in α-SMA- or vimentin-positive fibroblastic cells (Fig. 2a,b) but was not colocalized with CAM5.2-positive cancer cells (Fig. 2c). However, in non-neoplastic regions, miR-143 was highly expressed

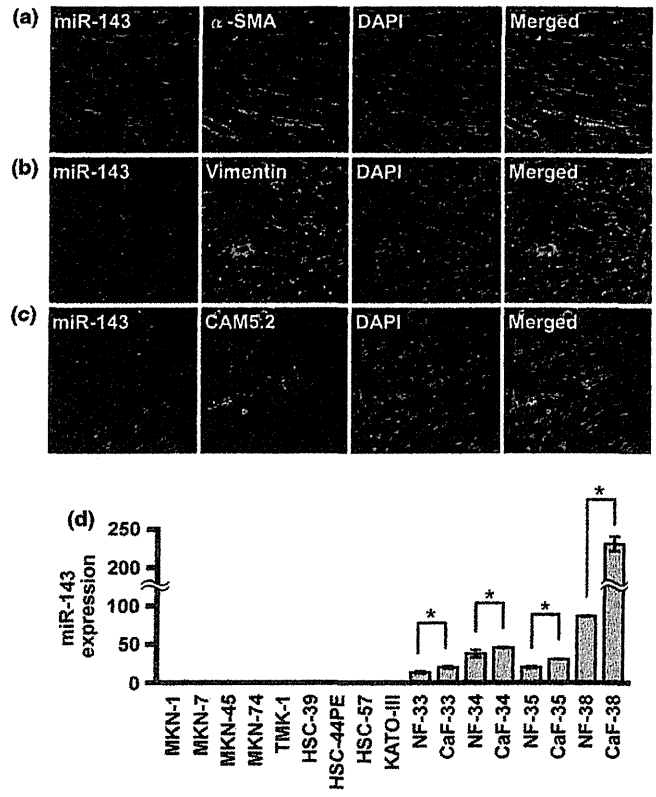

Fig. 2. MicroRNA-143 (miR-143) expression in scirrhous type gastric cancer (GC) tissue and cell lines. *In situ* hybridization of miR-143 was carried out in combination with immunofluorescence staining in scirrhous type GC. MicroRNA-143 labeling was revealed by Cy3-conjugated streptavidin (red). (a) α-Smooth muscle actin (α-SMA), (b) vimentin, or (c) CAM5.2 labeling was revealed by FITC-conjugated secondary antibody (green). DNA was counterstained with DAPI (blue). MicroRNA-143 expression was localized to α-SMA-positive and vimentin-positive stromal fibroblasts. (d) MicroRNA-143 expression levels were evaluated in GC cell lines and fibroblasts. Bars and error bars indicate median and standard error, respectively. $*P < 0.05$. CaF, cancer-associated fibroblasts; NF, normal gastric fibroblasts.

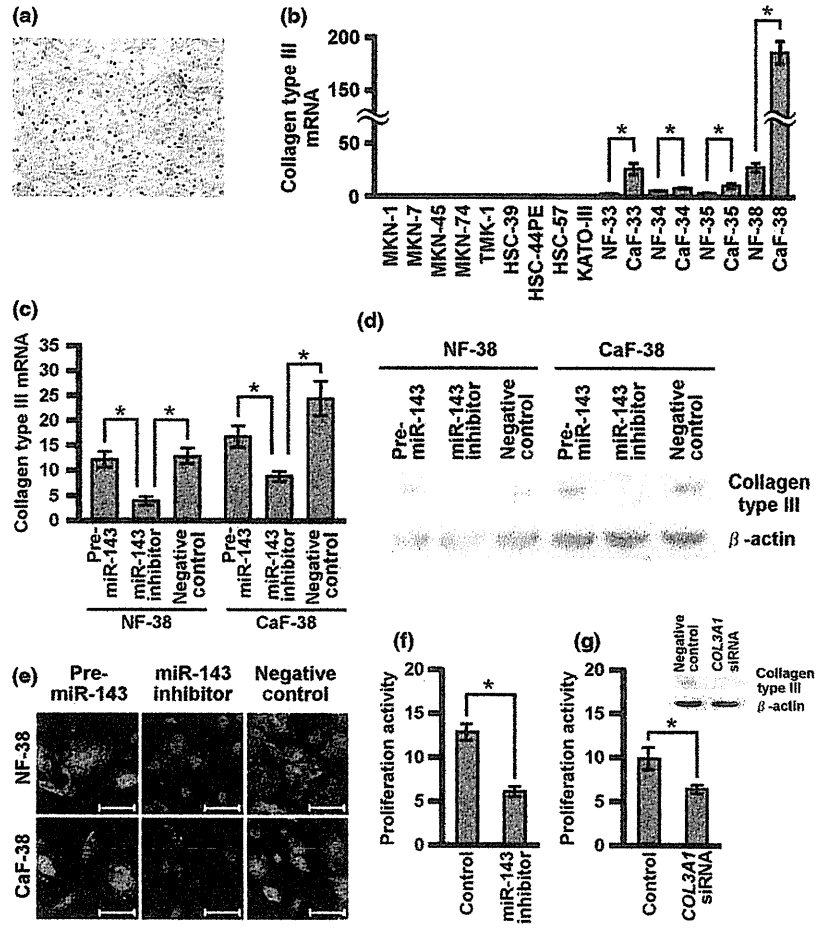

Fig. 3. Regulation of collagen type III expression by microRNA-143 (miR-143). (a) Collagen type III expression was assessed by immunohistochemical analysis of scirrhous type gastric cancer (GC) tissue. (b) Collagen type III mRNA expression levels were evaluated in GC and fibroblasts. Normal gastric fibroblasts and cancer-associated fibroblasts (NF-38 and CaF-38, respectively) were transfected with negative control miRNA or precursor miR-143 or miR-143 inhibitor, and (c) quantitative RT-PCR, (d) Western blot, and (e) cell staining were carried out for collagen type III expression. Scale bars: 50 μm. (f) Proliferation activity after coculture of HSC-44PE GC cells and CaF-38 with miR-143 inhibitor or negative control. Proliferation activity of HSC-44PE was assessed by percentages of BrdU /CAM5.2-positive cells. (g) Proliferation activity after coculture of HSC-44PE and CaF-38 with *COL3A1* siRNA or negative control. Collagen type III expression level was determined by Western blot analysis. Results are mean ± SE of triplicate measurements. $^*P < 0.05$.

in normal epithelial cells, but the expression was faint or not present in stromal fibroblasts in non-neoplastic tissue (data not shown). Expression of miR-143 was also examined in 9 GC cell lines, as well as in NFs and CaFs. Expression of miR-143 was evident in NFs and CaFs, but was undetectable in GC cell lines (Fig. 2d). Moreover, the expression levels of miR-143 were higher in CaFs than in NFs, and CaFs derived from scirrhous type GC showed a tendency toward higher expression of miR-143 (Fig. 2d). These data indicated that miR-143 is localized to epithelial cells in normal gastric tissue, but its localization is changed to surrounding stromal fibroblasts, but not cancer cells, in scirrhous type GC.

MicroRNA-143 regulates collagen type III expression in stromal fibroblasts of scirrhous type GC. Because miR-143 was found to be expressed in stromal fibroblasts but not in cancer cells, we sought to investigate the function of miR-143 in stromal fibroblasts. Scirrhous type GC produces abundant collagen and thus promotes fibrosis.[31,32] We previously reported that collagen type III expression is associated with scirrhous type GC.[6,7] We first examined collagen type III expression in scirrhous type GC tissue by immunostaining and, as expected, collagen type III was detected in fibrillar bundles of scirrhous type GC (Fig. 3a). Collagen type III mRNA expression was examined in GC and fibroblasts by qRT-PCR. High levels of collagen type III mRNA expression were observed in stromal fibroblasts that retained high miR-143 expression (Fig. 3b). To assess the relation between collagen type III and miR-143, NF-38 and CaF-38 were selected because they had the highest miR-143 and collagen type III mRNA expression (Fig. 3b). Transfection of miR-143 inhibitor significantly suppressed collagen type III expression (Fig. 3c–e). In contrast, transfection of miR-143 precursor sustained or increased collagen type III expression (Fig. 3c–e). These data suggest that miR-143 positively regulates collagen type III expression in stromal fibroblasts of scirrhous type GC.

To address the biological significance of collagen type III induction by miR-143 in CAFs, HSC-44PE was directly cocultured with CaF-38 that was treated with miR-143 inhibitor or collagen type III siRNA. We used HSC-44PE because it was derived from scirrhous type GC patients. Proliferation of HSC-44PE cells was significantly repressed in coculture with miR-143- or collagen type III-inhibited CaF-38 (Fig. 3f,g). These data indicated that both miR-143 and collagen type III expression in stromal fibroblast could affect cancer cell proliferation.

Transforming growth factor-β regulates collagen type III expression through miR-143. Scirrhous type GC secretes a larger amount of active form TGF-β than non-scirrhous type GC does,[32] and TGF-β has an important pathological and biological role in scirrhous type GC.[5,7,33,34] To investigate the effect of TGF-β1 on miR-143 and collagen type III expression, NF-38 and CaF-38 were treated with TGF-β1, and their miR-143 expression levels were monitored by qRT-PCR. There were no differences in the expression of endogenous TGF-β1, TGF-β2, or TGF-β3 between NF-38 and CaF-38 (Fig. S2). Treatment with TGF-β1 resulted in strong induction of miR-143 and collagen type III mRNA expression within 96 h in