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

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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, 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
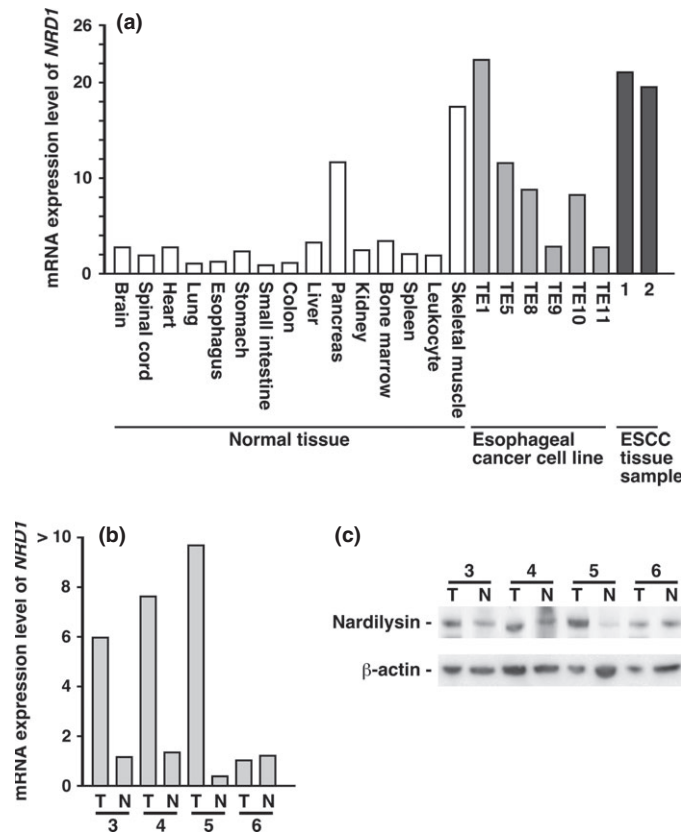

Fig. 1. Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis of *NRD1* mRNA and Western blot analysis of nardilysin protein in normal tissue samples, esophageal cancer cell lines, and esophageal squamous cell carcinoma (ESCC) tissue samples. (a) mRNA expression level of *NRD1* 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
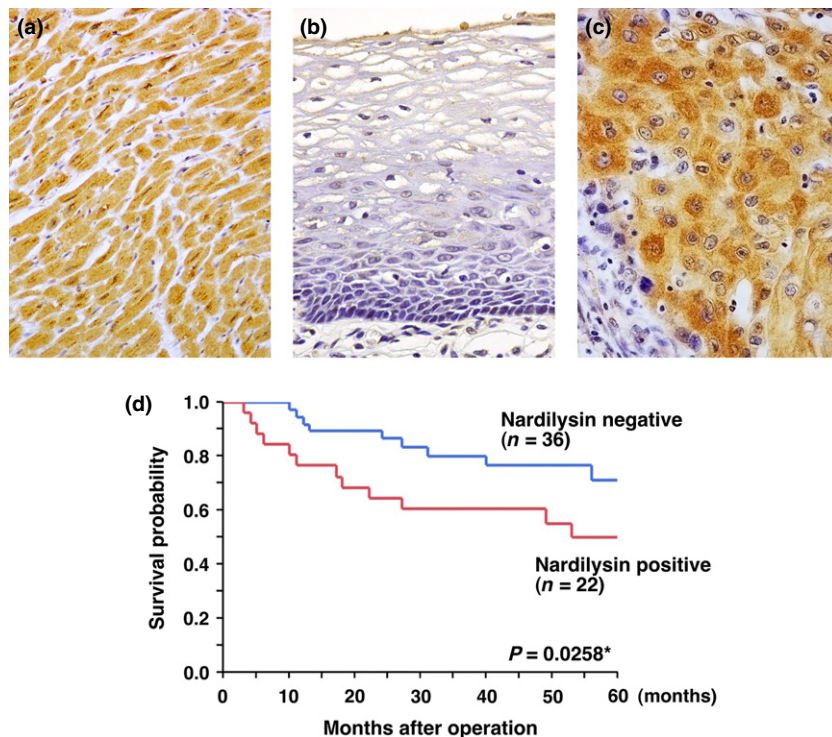
Fig. 2. Immunohistochemical analysis of nardilysin in normal tissue and esophageal squamous cell carcinoma (ESCC) tissue samples. (a) Immuno-histochemical analysis of nardilysin in normal skeletal muscle (original magnification: ×200). (b) Immunohistochemical analysis of nardilysin in non-neoplastic 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

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.

independent prognostic predictor for survival in patients with ESCC (HR 2.476; 95% CI 1.039–6.115; $P = 0.0407$).

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		2.476	
	(1.087–6.313)		(1.039–6.115)	
Tumor stage				
0/I/II	1 (Ref.)	<0.0001	1 (Ref.)	<0.0001
III/IV	8.919		8.895	
	(3.585–25.215)		(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/	1.064			
Poorly	(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
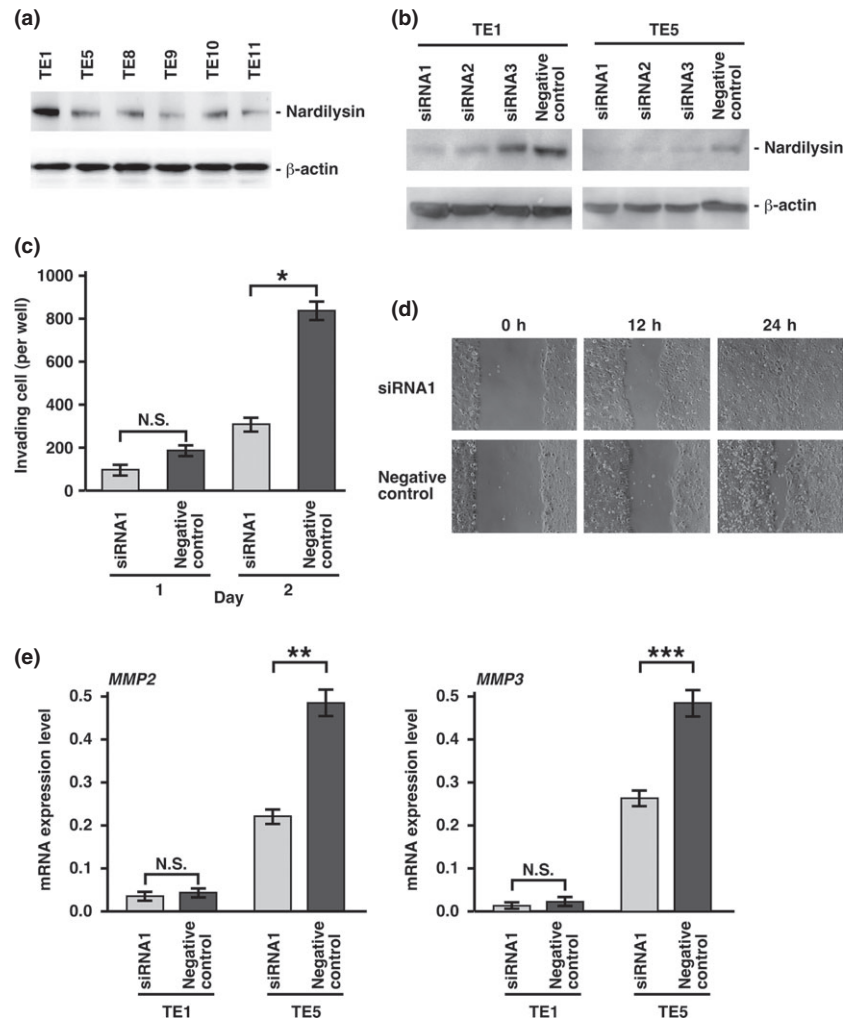
Fig. 3. Functional analysis of nardilysin in esophageal cancer cell lines. (a) Western blot analysis of nardilysin in six esophageal cancer cell lines. (b) Western blot analysis of nardilysin in cell lysates from TE1 and TE5 cells transfected with *NRD1* siRNA (siRNA1–3) and negative control siRNA. (c) 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 chemo-therapy. Further analysis will help to elucidate the biological function and prognostic value of nardilysin in ESCC.

Disclosure Statement

The authors have no conflict of interest.

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