ANTICANCER RESEARCH *41*: 609-617 (2021)
doi:10.21873/anticanres.14812

Tissue *RNFT2* Expression Levels Are Associated With Peritoneal Recurrence and Poor Prognosis in Gastric Cancer

MASAHIRO SASAHARA, MITSURO KANDA, DAI SHIMIZU, CHIE TANAKA, YOSHIKUNI INOKAWA, NORIFUMI HATTORI, MASAMICHI HAYASHI, GORO NAKAYAMA and YASUHIRO KODERA

Department of Gastroenterological Surgery (Surgery II), Nagoya University Graduate School of Medicine, Nagoya, Japan

Abstract. *Background/Aim: Disease recurrence is frequently observed after curative resection of advanced gastric cancer resulting in a poor prognosis. In the present study, we identified a candidate biomarker to predict recurrence and prognosis after curative resection of gastric cancer. Materials and Methods: A transcriptome analysis was conducted using surgically resected cancerous tissue from patients with metastatic gastric cancer to identify genes that are upregulated in primary and metastatic tissues. Results: Ring finger protein, transmembrane 2 (RNFT2) mRNA expression was upregulated in primary gastric cancer tissues and metastases compared with non-cancerous tissues. RNFT2 expression in gastric cancer cell lines was positively correlated with the EMT-related molecules GSC, MMP9, and RAC1. The RNFT2 high expression group exhibited a significantly shorter postoperative overall survival. Peritoneal recurrence was significantly higher in the RNFT2 high expression group. Conclusion: RNFT2 mRNA expression predicts peritoneal recurrence and is a potential prognostic biomarker for gastric cancer following curative gastrectomy.*

Gastric cancer has become the leading cause of cancer-related deaths worldwide (1). Despite advances in our understanding of the pathogenesis and treatment of gastric cancer, the recurrence rate after radical gastrectomy remains high and prognosis is poor (2, 3). The identification of biomarkers that predict gastric cancer metastasis and recurrence will enable physicians to predict the risk of recurrence in individual patients (4, 5). Perioperative chemotherapy and postoperative follow-up, considering the respective risks, may contribute to the prevention of recurrence, early detection, and improved prognosis.

The type of recurrence and metastasis of gastric cancer include lymph node recurrence, hematogenous metastasis and peritoneal dissemination. Among them, in particular peritoneal metastasis is difficult to detect early and systemic chemotherapy is largely ineffective. As a result, peritoneal metastasis is a major cause of poor prognosis in gastric cancer (6).

Defining the risk of peritoneal metastasis is necessary for prediction and early detection, but there are currently no established biomarkers.

To identify differentially expressed genes that associate with the metastasis of gastric cancer, we conducted a transcriptome analysis of gastric clinical specimens from patients with distant metastasis. We identified a ring finger protein, transmembrane 2 (*RNFT2*), as a novel candidate biomarker. *RNFT2* has been implicated in mediating protein interactions and the ubiquitination of target proteins, but its role in gastric cancer has not been reported. In the present study, we investigated the relationship between *RNFT2* mRNA expression in gastric cancer tissues and the likelihood of recurrence after curative gastrectomy.

Materials and Methods

Ethics. This study conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects and was approved by the Institutional Review Board of Nagoya University, Japan (Approval Number 2014-0043). Written informed consent for the use of clinical samples and data was obtained from all patients.

Transcriptome analysis. Surgically resected gastric and metastatic tissues from four patients with hepatic metastasis were subjected to transcriptome analysis. Global expression profiling was conducted using the Illumina HiSeq platform (San Diego, CA, USA) to evaluate the expression of 57,749 genes in primary gastric cancer tissues compared with corresponding noncancerous adjacent gastric mucosa.

Cell lines. Fourteen gastric cancer cell lines (AGS, GCIY, IM95,

This article is freely accessible online.

Correspondence to: Mitsuro Kanda, MD, Ph.D., Department of Gastroenterological Surgery (Surgery II), Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. Tel: +81 527442249, Fax: +81 527442255, e-mail: m-kanda@med.nagoya-u.ac.jp

Key Words: Gastric cancer, *RNFT2*, prognosis, peritoneal recurrence.

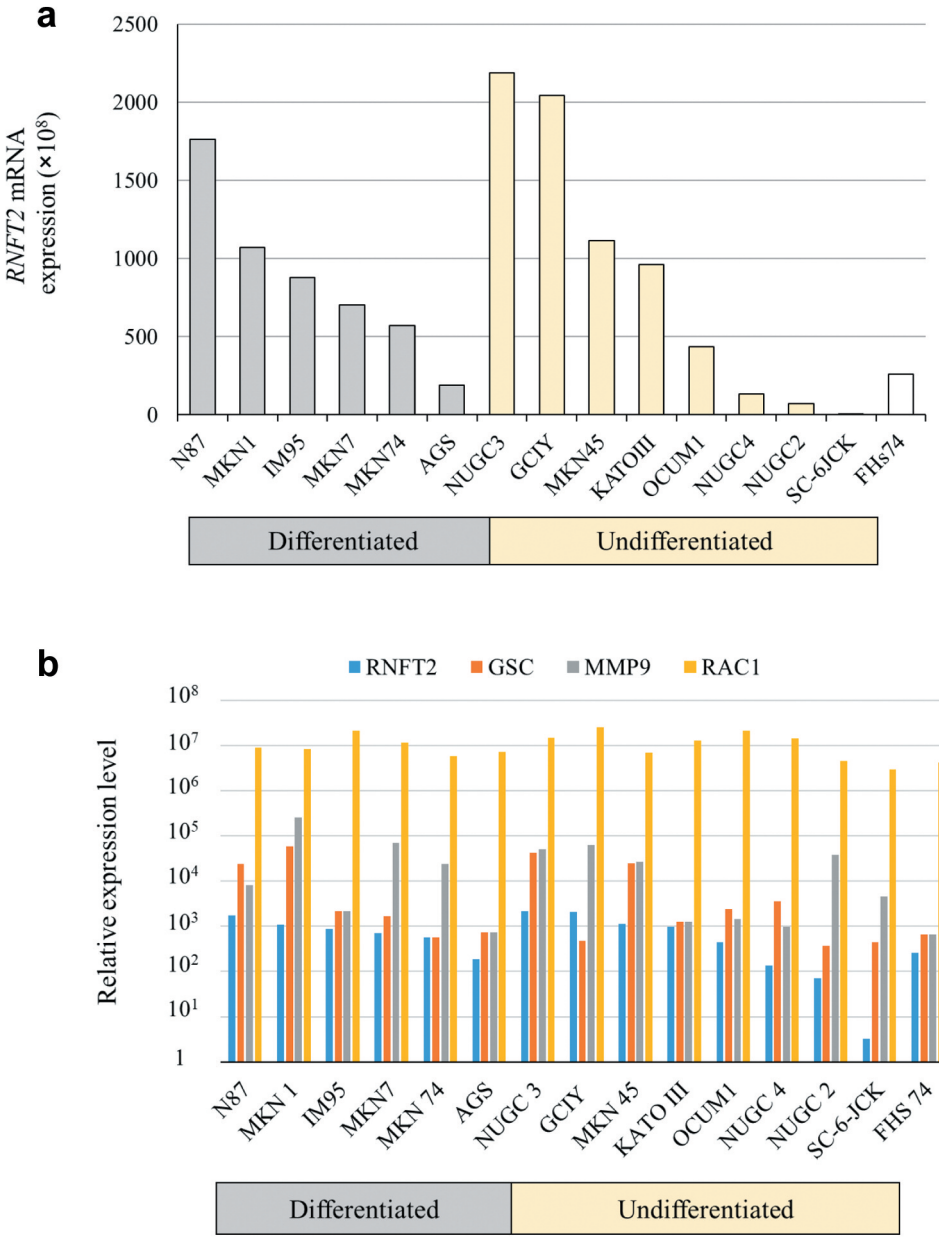

Figure 1. *Continued*

KATOIII, MKN1, MKN7, MKN45, MKN74, N87, NUGC2, NUGC3, NUGC4, OCUM1, and SC-6-JCK) and a non-tumorigenic epithelial cell line (FHs74) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) or the Japanese Collection of Research Bioresources Cell Bank (JCRB; Osaka, Japan). The cell lines were cultured in RPMI-1640 (Sigma- Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum at 37˚C in an atmosphere containing 5% CO_2.

Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) and PCR array analysis. Total RNA (10 μg per sample) was isolated from cells and tissues and used to generate complementary DNA (cDNA) and amplified using gene-specific PCR primers. Primers for the *RNFT2* were as follows: forward 5'-GAAAGGACTCCCCTTCATCC-3' and reverse 5'-CCAGCA CTGACCTCTTCTCC-3'. Real-time detection of SYBR® Green fluorescence intensity was performed using the ABI StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression was used as an internal standard. The qRT-PCR were performed in triplicate for each sample. Expression levels are presented as the value for *RNFT2* divided by that of *GAPDH*. The expression levels of 84 additional genes were analyzed using the Human Epithelial to Mesenchymal Transition (EMT) RT Profiler

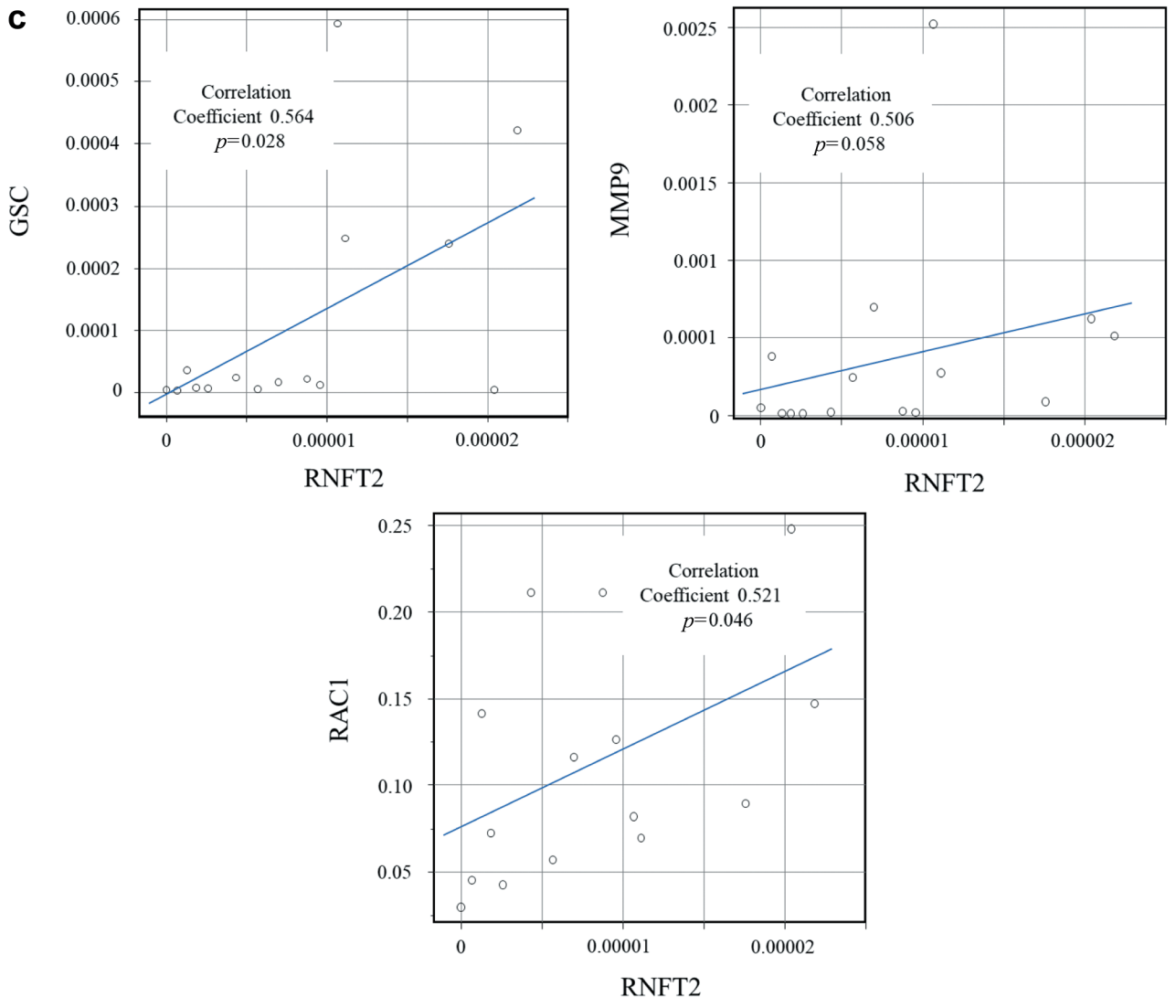

Figure 1. *Analysis of RNFT2 expression in 14 gastric cancer cell lines and EMT-related genes. (a) Quantitative RT-PCR analysis of RNFT2 mRNA expression in gastric cancer cell lines and a control cell line. (b) PCR array analysis of RNFT2 and EMT-related genes. (c) Correlation analysis between RNFT2 and GSC, MMP9, and RAC1.*

PCR Array (Qiagen, Hilden, Germany) and correlated to the expression of *RNFT2* to identify genes associated with *RNFT2* in gastric cancer cell lines.

Collection of clinical samples. Between 2001 and 2017, primary gastric cancer tissues and corresponding normal adjacent tissues were collected from 230 patients who underwent radical gastrectomy for gastric cancer without neoadjuvant therapy at the Department of Gastroenterological Surgery, Nagoya University Hospital. All patients were classified histologically in accordance with the 8th edition of the Union for International Cancer Control (UICC) classification (7). Tissue samples were immediately frozen in liquid nitrogen and stored at –80˚C. Tumor samples without necrotic areas were extracted by gross observation. Corresponding normal adjacent gastric mucosa samples >5 cm from the edge of the tumors were obtained from the same patient. Patients were pathologically diagnosed with stages I-III gastric cancer, and relevant clinicopathological parameters were acquired from patient medical records. Since 2006, adjuvant chemotherapy using S-1 (an oral fluorinated pyrimidine) was administered to all UICC stage II-III patients with gastric cancer, unless contraindicated by the patient's condition (8).

Evaluation of the clinical significance of RNFT2 expression. *RNFT2* mRNA levels were measured in 230 matched pairs of resected gastric tissues. For external validation of the data, a freely available integrated dataset comprising 1065 patients from three major cancer research centres (Berlin, Bethesda and Melbourne datasets) was accessed at http://kmplot.com/analysis/ (9).

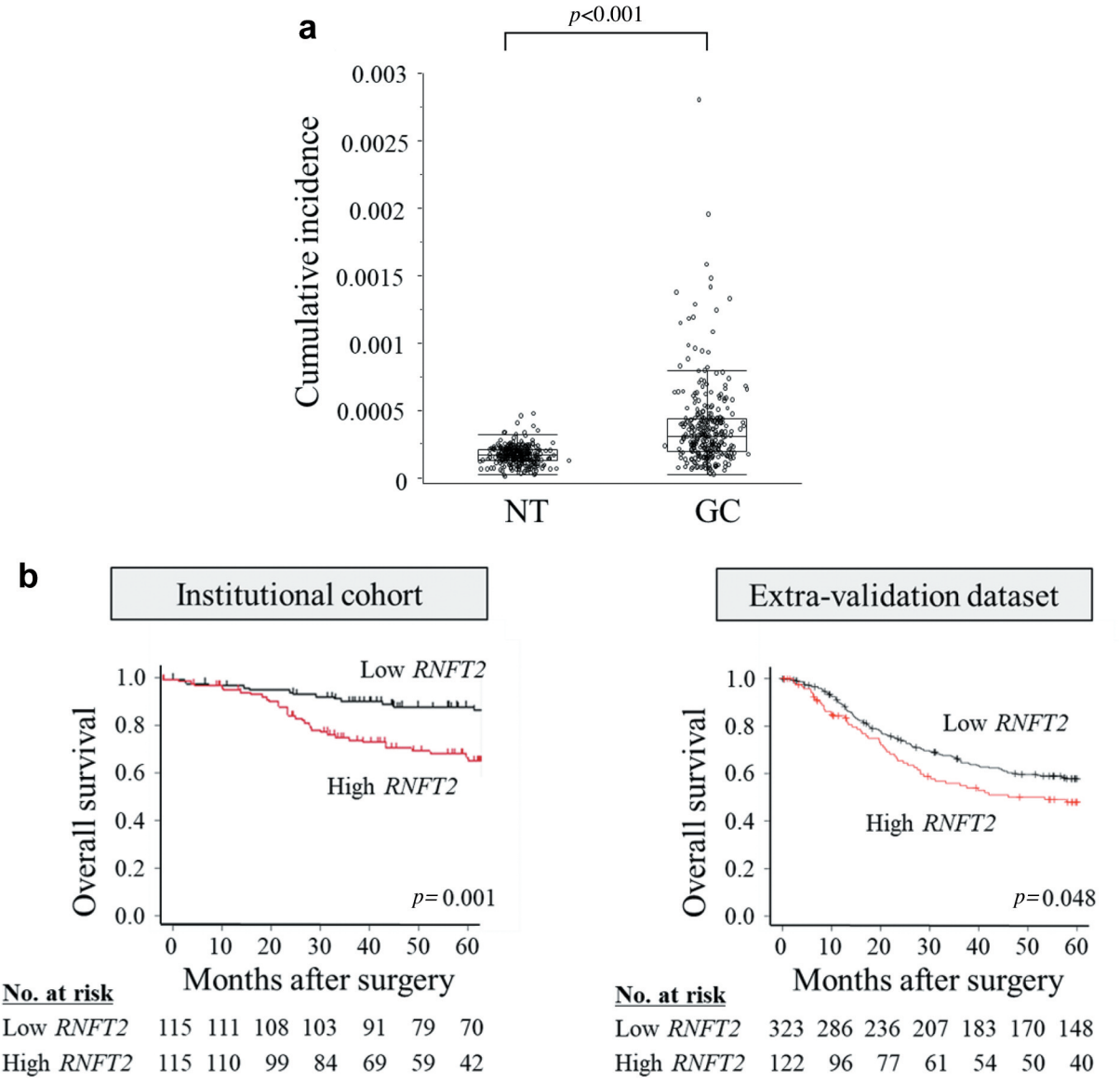

Figure 2. *RNFT2 mRNA expression in gastric cancer and normal tissues and its correlation with prognosis. (a) Comparison of RNFT2 mRNA expression in gastric cancer (GC) tissues and normal adjacent tissues (NT). (b) Overall survival time of 230 patients with stage I-III gastric cancer and an extra-validation dataset, according to high and low RNFT2 expression.*

Statistical analysis. Relative levels of mRNA expression (*RNFT2*/*GAPDH*) between gastric cancer and adjacent normal tissues were examined using the Mann–Whitney *U*-test. The χ^2 test was used to analyze the significance of the association between *RNFT2* expression and clinicopathological parameters. The significance of the correlation between *RNFT2* and EMT-related genes was assessed using Spearman's rank correlation coefficient.

Overall survival and disease-free survival rates were calculated using the Kaplan–Meier method, and the difference in survival curves was analyzed using the log-rank test. Multivariate regression analysis was performed to detect prognostic factors using the Cox proportional hazards model and variables with $p<0.05$ were entered into the final model. All statistical analyses were performed using JMP 15 software (SAS Institute Inc., Cary, NC, USA). A value of $p<0.05$ was considered statistically significant.

Results

Identification of RNFT2 as a candidate gastric cancer-related gene. We conducted a comprehensive expression analysis of primary cancerous tissues obtained from patients with concurrent hepatic metastasis. We compared the mRNA expression levels in normal tissues, cancerous tissues, and

Table I. *RNFT2 expression and clinical characteristics of 230 gastric cancer patients.*

Variables	Low *RNFT2* (n=115)	High *RNFT2* (n=115)	*p*-Value
Age			
<70 year	75	63	0.106
≥70 year	40	52	
Gender			
Male	77	88	0.107
Female	38	27	
CEA (ng/ml)			
≤5	101	94	0.196
>5	14	21	
CA19-9 (IU/ml)			
≤37	95	96	0.861
>37	20	19	
Tumor location			
Entire	4	4	0.227
Upper third	24	37	
Middle third	41	39	
Lower third	46	35	
Tumor size (mm)			
<50	60	64	0.597
≥50	55	51	
Macroscopic type			
Borrmann type 4/5	11	11	1.000
Others	104	104	
Multifocal lesions			
Absent	104	105	0.819
Present	11	10	
Tumor depth (UICC)			
pT1-3	76	71	0.492
pT4	39	44	
Differentiation			
Differentiated	50	49	0.894
Undifferentiated	65	66	
Lymphatic involvement			
Absent	21	17	0.477
Present	94	98	
Vascular invasion			
Absent	51	37	0.057
Present	64	78	
Infiltrative growth type			
Invasive growth	37	34	0.669
Expansive growth	78	81	
Lymph node metastasis			
Absent	53	34	0.010
Present	62	81	
UICC stage			
I	27	23	0.346
II	39	32	
III	49	60	

CEA: Carcinoembryonic antigen; CA19-9: carbohydrate antigen 19-9; UICC: Union for International Cancer Control.

hepatic metastases, and identified genes that were overexpressed in both primary and metastatic cancerous tissues compared with normal tissues. Among them, we selected *RNFT2* for the following reasons: no previous studies on *RNFT2* mRNA expression in gastric cancer have been reported, primers suitable for qPCR assay can be designed, and expression analysis was feasible in a pilot study using a small number of clinical specimens. A transcriptome analysis revealed that *RNFT2* expression was upregulated in primary gastric cancer tissues ($\log_2$ 1.034) and metastases ($\log_2$ 3.187) compared with non-cancerous areas. *RNFT2* has been reported to mediate protein interactions and participate in the ubiquitination of target proteins, however, a thorough understanding of its precise function remains to be established. Therefore, we focused on *RNFT2* which may be associated with the malignancy of gastric cancer.

Analysis of RNFT2 mRNA expression in gastric cancer cell lines. To characterize *RNFT2* in gastric cancer, we evaluated *RNFT2* mRNA expression in 14 gastric cancer cell lines compared with control epithelial cells. *RNFT2* expression was higher in 10 gastric cell lines (N87, MKN1, IM95, MKN7, MKN74, NUGC3, GCIY, MKN45, KATO-III and OCUM1) compared with FHs74 control cells (Figure 1a). *RNFT2* mRNA levels were similar between differentiated and undifferentiated carcinomas.

A PCR array analysis of 84 EMT-related genes identified three EMT-related genes: goosecoid homeobox (GSC), matrix metalloproteinase 9 (MMP9) and rac family small GTPase1 (RAC1) whose expression correlated with *RNFT2* expression (Spearman coefficient ≥0.5) (Figure 1b and c).

Expression levels of RNFT2 mRNA in surgically resected gastric tissues. We examined the expression of *RNFT2* mRNA in cancerous and non-cancerous tissues from 230 patients who underwent radical gastrectomy. The patient population included 165 men and 65 women aged 26 to 96 years (66.2±10.8 years, mean±standard deviation). Pathologically, 99 and 131 patients were diagnosed with differentiated and undifferentiated gastric cancer, respectively. According to the UICC staging system (eighth edition), 50, 71 and 109 patients exhibited the characteristics of pathological stages I, II and III, respectively. The expression of *RNFT2* mRNA was significantly higher in cancerous tissues compared with that of non-cancerous adjacent tissues ($p<0.001$; Figure 2a).

Prognostic implications of RNFT2 mRNA expression levels. We categorized patients into high (above the median *RNFT2* mRNA expression) and low *RNFT2* expression groups. The high *RNFT2* expression group exhibited a significant correlation with positive lymph node metastasis, but there was no difference in age, sex, degree of differentiation, or UICC stage (Table I). The overall survival rate was significantly shorter in the high *RNFT2* expression group compared with the low *RNFT2* expression group, and extra-validation data showed a similarly poor prognosis and reproducibility in the

Table II. *Prognostic factors for disease-free survival in 230 gastric cancer patients.*

Variables	n	Univariate			Multivariable		
		Hazard ratio	95%CI	*p*-Value	Hazard ratio	95%CI	*p*-Value
Age (≥70)	92	0.82	0.49-1.39	0.420			
Gender (female)	65	0.95	0.57-1.58	0.834			
CEA (>5 ng/ml)	35	1.27	0.68-2.36	0.453			
CA 19-9 (>37 IU/ml)	39	2.35	1.37-4.03	0.002	1.70	0.98-2.98	0.060
Tumor location (lower third)	81	0.76	0.46-1.27	0.297			
Tumor size (≥50 mm)	106	1.95	1.21-3.15	0.006	1.41	0.87-2.30	0.168
Macroscopic type (Borrmann type 4/5)	22	2.32	1.27-4.24	0.007	1.32	0.679-2.59	0.410
Multifocal lesions	21	0.90	0.39-2.09	0.816			
Tumor depth (pT4, UICC)	83	2.55	1.59-4.08	<0.001	1.50	0.88-2.55	0.139
Tumor differentiation (undifferentiated)	131	1.59	0.97-2.60	0.066			
Lymphatic involvement	192	4.12	1.50-11.3	0.006	0.95	0.30-3.07	0.936
Vascular invasion	142	2.66	1.52-4.65	<0.001	1.34	0.72-2.49	0.348
Invasive growth	71	1.66	1.03-2.69	0.038	1.22	0.69-2.14	0.501
Lymph node metastasis	143	7.97	3.63-17.5	<0.001	4.82	1.96-11.8	<0.001
High *RNFT2*	115	2.14	1.31-3.50	0.002	1.85	1.12-3.06	0.016

CI: Confidence interval; CEA: carcinoembryonic antigen; CA19-9: carbohydrate antigen 19-9; UICC: Union for International Cancer Control.

high *RNFT2* expression group (5-year survival rates, 67% and 87%, respectively, $p<0.001$; Figure 2b).

To identify prognostic factors, we performed a univariate analysis for disease-free survival. Carbohydrate antigen (CA) 19-9 >37 IU/ml, tumor size (≥50 mm), macroscopic type (Borrman type 4/5), pT4, lymphatic involvement, vascular invasion, invasive growth, lymph node metastasis, and high *RNFT2* mRNA expression were detected as significant poor prognostic factors. Multivariate analysis of disease-free survival revealed that high *RNFT2* expression and lymph node metastasis were significantly independent poor prognostic factors (Table II).

Disease-free survival was significantly shorter in the high *RNFT2* expression group compared with the low *RNFT2* expression group (5-year, disease-free survival rates, 61% and 80%, respectively, $p=0.002$; Figure 3a). The frequency of peritoneal recurrence was significantly higher in the high *RNFT2* expression group compared with the low *RNFT2* expression group (Figure 3b). The cumulative incidence of peritoneal recurrence was significantly higher in the high *RNFT2* group compared with the low *RNFT2* expression group (Figure 3c).

Discussion

In the present study, we identified a novel gene that may serve as a biomarker for metastasis and recurrence in gastric cancer. We performed a comprehensive expression analysis of gastric tissues from patients with hepatic metastasis and identified the *RNFT2* gene as highly expressed in cancerous and metastasis tissues compared with non-cancerous tissues. *RNFT2* expression levels were positively correlated with several EMT-related molecules. The high *RNFT2* mRNA expression group exhibited a high recurrence rate after curative gastrectomy, especially peritoneal recurrence, and shorter overall survival. This result was also observed in the extra-validation dataset.

Human *RNFT2*, located on chromosome 12q24.22, encodes a 48,965-Da protein consisting of 444 amino acids. *RNFT2* is a member of the ring finger family and has been implicated in proteolysis in the cytosol and nucleus. It is highly expressed in the brain and testis and ubiquitously expressed in multiple organs including the gastrointestinal tract, liver, adrenal gland, and lung. *RNFT2* has been reported to decrease lung inflammation and damage by ubiquitinating and degrading IL-3Ra, the receptor for the IL-3 inflammatory cytokine (10). There have been no reports of an association between malignancy and *RNFT2* and only a few reports suggest that *RNFT1* is involved in the suppression of metastasis in breast cancer (11). To our knowledge, this is the first study to evaluate the association between *RNFT2* and gastric cancer.

We conducted a PCR array analysis to identify genes that are associated with *RNFT2* expression and metastasis in gastric cancer. *RNFT2* was positively correlated with the expression of the EMT-related molecules GSC, MMP9, and RAC1. EMT is an important process that regulates the migration and invasion of cancer cells and is required for the development of metastatic lesions. EMT involves a complex group of molecules consisting primarily of transcription factors (12). MMP9 changes epithelial cells into mesenchymal cells by degrading the extracellular matrix and basement membrane and causing a loss of epithelia integrity, which results in a loss of adhesion to surrounding cells. One of the features of EMT is decreased expression of E-cadherin. MMP9 degrades epithelial E-cadherin, which results

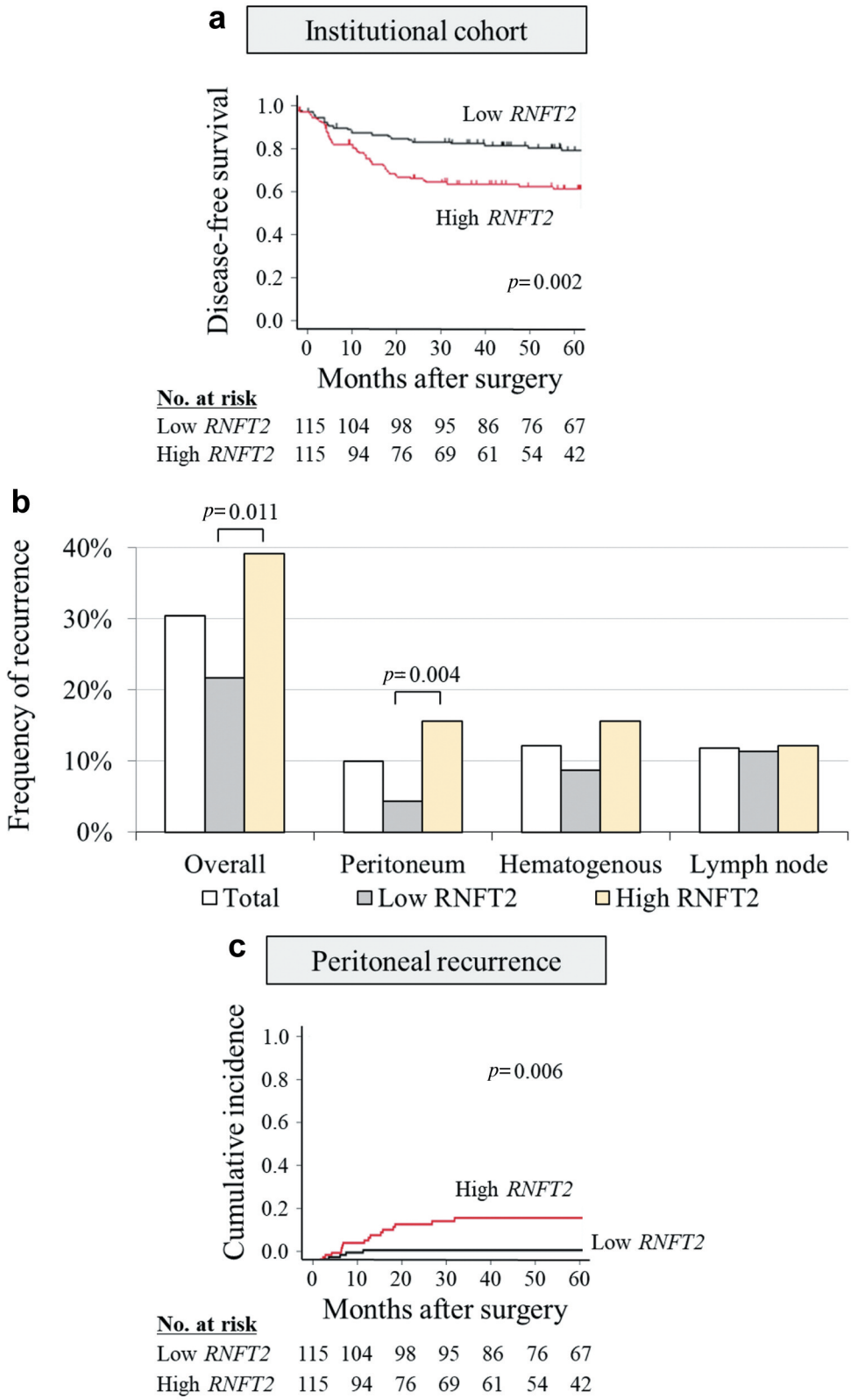

Figure 3. *Clinical significance of RNFT2 mRNA expression. (a) Disease-free survival time in 230 patients with stage I–III gastric cancer according to high and low RNFT2 expression levels. (b) Frequency of initial recurrence sites after curative gastrectomy in patients according to RNFT2 mRNA expression. (c) Cumulative incidence of peritoneal recurrence.*

in the dissociation of β-catenin from the adherens junction and its transfer to the nucleus. β-catenin accumulates in the nucleus and functions as a transcription factor to activate target genes of the Wnt signalling pathway that promote cell growth and metastasis (13). In breast cancer, MMP9 has been reported to be involved in angiogenesis and cancer cell migration (14). RAC1 is a member of the Rho family of G proteins, which are involved in cell proliferation, cytoskeleton formation, and tumor growth by activating the PI3k/Akt signalling pathway (15). Over-expression of GSC induces EMT and promotes tumor migration and invasion. In HCC, over-expression of GSC has been shown to be associated with distant metastasis and shorter survival (16). In breast cancer, GSC expression has been reported to be associated with metastasis (17). These associations suggest a molecular mechanism for *RNFT2* in metastasis and the recurrence of gastric cancer.

Currently in clinical practice, classic serum tumor markers (*e.g.*, carcinoembryonic antigen (*CEA*) and CA19-9) are used as biomarkers to determine the malignancy of gastric cancer (18, 19). However, in the present study, we did not observe an association between preoperative serum CEA levels and overall survival. Elevated CA19-9 levels were significantly associated with disease-free survival in a univariate analysis, but were not associated with disease-free survival in a multivariate analysis. We analyzed patients with stage I-III gastric cancer after curative resection. We considered the patients with stage IV gastric cancer to be unsuitable for the analysis of the correlation between *RNFT2* mRNA expression and prognosis because stage IV encompasses a wide range of cases, from only microscopic free cancer cells in the abdominal cavity (CY1P0H0M0) to systemic multiple metastases. Increased *RNFT2* expression was an independent poor prognostic factor and was considered superior to classic serum tumor markers as a prognostic biomarker after curative resection.

High levels of *RNFT2* expression in primary cancerous tissue predicted a high risk of recurrence, in particular, peritoneal recurrence. Peritoneal recurrence is a problem because of the difficulty of early detection by computed tomography (20). Nakanishi *et al*. have analyzed mRNAs in peritoneal lavage fluid samples and reported that synaptotagmin XIII (*SYT13*) and *CEA* levels may be predictive factors of peritoneal recurrence after curative resection (21). The combination of these molecular markers resulted in a more accurate risk assessment of patients with high *RNFT2* expression and may lead to improved precision medicine (21, 22).

Considering our results, in cases of high *RNFT2* expression in preoperative biopsy specimens, staging laparoscopy may be recommended to search for gross peritoneal dissemination and peritoneal lavage cytology to identify microscopic free cancer cells in the abdominal cavity (23). Large clinical trials have shown that postoperative adjuvant chemotherapy with an S-1 based regimen (S-1 monotherapy, docetaxel plus S-1) reduces postoperative peritoneal recurrence (24, 25). Because of the high risk for peritoneal recurrence in patients with high *RNFT2* expression, S-1-based chemotherapy may be recommended. During postoperative surveillance, increased attention should be given to abdominal symptoms and periodic abdominal ultrasonography should be performed to search for ascites to detect peritoneal recurrence at an early stage. If increased ascites is observed, a staging laparoscopy may be recommended to make a definitive diagnosis.

Our study has several limitations. First, expression analyses were performed retrospectively at a single institution. Second, the study subjects were acquired over a long time period making it impossible to eliminate potential bias. Third, although we adopted a median cut-off value for *RNFT2* mRNA expression, our results may improve if a cut-off value with higher sensitivity and specificity is identified. Last, functional analyses are necessary to clarify the underlying mechanism of the effects of *RNFT2* on the pathology of gastric cancer.

Conclusion

We demonstrated that high *RNFT2* mRNA expression in gastric cancer tissue is an independent poor prognostic factor following curative resection. *RNFT2* may represent a useful biomarker for predicting peritoneal recurrence and prognosis.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

MS and MK conceived the study concept and design, analyzed data and wrote the manuscript. MK, DS and YK contributed to data acquisition and interpretation. CT, IY, NH, MH, GN and YK revised the draft. All Authors have read and approved the final version of the manuscript.

Acknowledgements

The Authors thank Edanz Group (https://en-author-services.edanzgroup.com/ac) for editing a draft of this manuscript.

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Received December 15, 2020
Revised December 30, 2020
Accepted December 31, 2020