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Overexpression of *TRIM44* contributes to malignant outcome in gastric carcinoma

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Recent studies have shown that some members of the tripartite motif-containing protein (TRIM) family, which is characterized by a conserved RING finger, B-box, and coiled-coil domains, function as important regulators for carcinogenesis. In this study, we tested whether *TRIM44* (11p13) acts as a cancer-promoting gene through overexpression in gastric cancer. We analyzed seven gastric cancer cell lines and 112 primary tumors, which were curatively resected in our hospital between 2001 and 2003. Expression of the TRIM44 protein was detected in gastric cancer cell lines (2/7 cell lines; 29%) and primary tumor samples of gastric cancer (29/112 cases; 25%). Knockdown of TRIM44 expression using several specific siRNAs inhibited the proliferation, migration, and invasion of TRIM44-overexpressing cells. Overexpression of the TRIM44 protein was significantly correlated with an advanced type of macroscopic appearance, lymphatic invasion, and higher recurrence rate. TRIM44-overexpressing tumors had a worse overall rate of survival than those with non-expressing tumors ($P = 0.0038$, log–rank test) in both intensity and proportion expression-dependent manner. TRIM44 positivity was independently associated with worse outcome in multivariate analysis ($P = 0.0233$, hazard ratio 3.37 [1.18–9.64]). These findings suggest that TRIM44 plays a crucial role in tumor cell proliferation through its overexpression, and highlight its usefulness as a predictor and potential therapeutic target in gastric cancer. (*Cancer Sci* 2012; 103: 2021–2026)

Gastric cancer is the second leading cause of cancer-related death in the world.[1] Recent advances in diagnostic techniques and perioperative management have increased early detection of gastric cancer and decreased the mortality rate. However, patients with advanced disease still frequently develop recurrent disease despite extended radical resections, and consequently present extremely poor survival rates.[2]

Many genes have been analyzed in attempts to understand the molecular mechanism and improve clinical outcomes for human gastric cancers, however, only a few with frequent alterations have been identified.[3] Gene amplifications of *MET* and *ERBB2*, mutations of *TP53*, *APC*, and *E-cadherin*,[4,5] oncogenic activations of β-catenin and K-ras,[6,7] inactivation of the mismatch repair gene *hMLH1* associated with microsatellite instability,[8] and hypermethylation of *p16* are repeatedly reported.[9,10] As shown in these reports, studies have attempted to identify biological factors involved in the malignant potential of gastric cancer. However, in clinical settings, few genes have been assayed as therapeutic targets and/or diagnostic biomarkers, suggesting that novel genes associated with the progression of gastric cancer need to be identified.

The ubiquitin–proteasome system has crucial roles in physiology and pathophysiology.[11] Of the molecules associated with the ubiquitin–proteasome system, many RING finger ubiquitin E3 ligases are reported to be implicated in malignancy.[12–14] Recent studies have indicated that some members of tripartite motif (TRIM) proteins, which are characterized by a conserved RING finger, B-box, and coiled-coil domains, function as important regulators for carcinogenesis.[15] In this study, we tested whether TRIM-containing protein 44 (*TRIM44*: 11p13) acts as a cancer-promoting gene through activation/overexpression in gastric cancer. TRIM44 protein, which is a member of the TRIM protein family, was cloned from mouse brain cDNA library in 2001.[16] Until now, there were only five reports concerning TRIM44 and its gene function was not well clarified. High-level amplification in head and neck cancer[17] and gene expression identified by gene expression array in esophageal and junctional adenocarcinoma[18] were reported previously. However, to date, there has been no report on its clinical significance and functions that contribute to gastric carcinogenesis.

Consequently, we showed that TRIM44 was frequently overexpressed in gastric cancer cell lines and primary gastric cancers. Overexpression of TRIM44 was a poor prognosticator independent of other prognostic factors. Also, we showed that downregulation of TRIM44 expression suppressed cell proliferation, migration, and invasion in gastric cancer cell lines. Our results provided evidence that TRIM44 could be an important molecular marker for determining malignant properties and a target for molecular therapy in patients with gastric cancer.

Materials and Methods

Cell culture, drug treatment, and primary tissue samples. A total of seven gastric cancer cell lines, KatoIII, NUGC4, HGC27, MKN7, MKN28, MKN45, and MKN74, were used in this study. HGC27 cells were cultured in DMEM:F12 medium and the others in RPMI-1640 medium (Sigma, St. Louis, MO, USA). All media were purchased from Sigma, and supplemented with 100 mL/L FBS (Trace Scientific, Melbourne, Vic., Australia). All cell lines were cultured in 50 mL/L CO_2 at 37°C in a humidified chamber. Primary tumor samples of gastric cancer had been obtained from 112 consecutive gastric cancer patients, who underwent curative gastrectomy (R0 or R1) at the Division of Digestive Surgery, Department of Surgery, Kyoto Prefectural University of Medicine (Kyoto, Japan) between 2001 and 2003. The samples were embedded in paraffin after 24 h of formalin fixation. Relevant clinical and survival data were available for all patients. Written consent was always obtained in the formal style and after approval by the local ethics committee. None of these patients underwent endoscopic mucosal resection, palliative resection,

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preoperative chemotherapy, or radiotherapy, and none of them had synchronous or metachronous multiple cancers in other organs. Disease stage was defined in accordance with the International Union against Cancer TNM classification.[19] The median follow-up period for surviving patients was 55.6 months (range, 0.5–84.2 months).

Loss-of-function by siRNA and cell growth analysis. Loss-of-function screening was done using siRNAs targeting TRIM44 (M-017337-01-0005, siGENOME SMARTpool, Human TRIM44 (54765); Dharmacon, Lafayette, CO, USA) and the control (D-001210-01-05, siGENOME4 Non-Targeting siRNA #1; Dharmacon). Each siRNA (10 nmol/L) was transfected into gastric cancer cells using Lipofectamine RNAiMAX (Invitrogen, St Louis, MO, USA) according to the manufacturer's instructions. The knockdown of a target gene was confirmed by Western blot analysis. For measurements of cell growth, the number of viable cells at various time points after transfection was assessed by colorimetric water-soluble tetrazolium salt assay (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan).[20]

Transwell migration and invasion assays. Transwell migration and invasion assays were carried out in 24-well modified Boyden chambers (BD Transduction, Franklin Lakes, NJ, USA). The upper surface of 6.4 mm diameter filters with 8 μm pores was precoated with (invasion assay) or without (migration assay) Matrigel (BD Transduction). The siRNA transfectants (2×10^4 cells per well) were transferred into the upper chamber. Following 48 h of incubation, the migrated or invasive cells on the lower surface of filters were fixed and stained with the Diff-Quik stain (Sysmex, Kobe, Japan), and stained cell nuclei were counted directly in triplicate. We assessed invasive potential by calculating the number of cells, which is the ratio of the percentage invasion through the Matrigel-coated filters relative to the migration through the uncoated filters of test cells over that in the control counterparts.

Western blot analysis. Anti-TRIM44 rabbit polyclonal antibody (BC024031) was purchased from Proteintech Group (Chicago, IL, USA) and anti-GAPDH antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TRIM44 is an affinity purified rabbit polyclonal antibody raised against a recombinant protein of TRIM44. Cells were lysed and their proteins were extracted by M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, Rockford, IL, USA).

Immunohistochemistry. Tumor samples were fixed with 10% formaldehyde in PBS, embedded in paraffin, sectioned into 5-μm-thick slices, and subjected to immunohistochemical staining of the TRIM44 protein with the avidin–biotin–peroxidase method as described by Naoi *et al.*[21] In brief, after deparaffinization, endogenous peroxidases were quenched by incubating the sections for 20 min in 3% H_2O_2. Antigen retrieval was carried out by heating the samples in 10 mmol/L citrate buffer (pH 6.0) at 95°C for 60 min. After treatment with Block Ace (Dainippon Sumitomo Pharmaceutical, Osaka, Japan) for 30 min at room temperature, sections were incubated at 4°C overnight with an anti-TRIM44 (1:200) antibody. The avidin–biotin–peroxidase complex system (Vectastain Elite ABC universal kit; Vector Laboratories, Burlingame, CA, USA) was used for color development with diaminobenzidine tetrahydrochloride. Slides were counterstained with Mayer's hematoxylin. A formalin-fixed esophageal cancer cell line overexpressing TRIM44 (TE13), in which >50% of cells showed staining of each protein, was used as a positive control; a formalin-fixed gastric cancer cell line with low expression of TRIM44 (HGC27, data not shown) and TE13 staining without the TRIM44 antibody was included as a negative control. For scoring TRIM44 expression, the intensity (intensity score: 0, negative; 1, weak; 2, moderate; 3, strong) and percentage of the total cell population (proportion score: $0 < 10\%$; $10\% \leq 1 \leq 33\%$; $34\% \leq 2 \leq 66\%$; $67\% \leq 3 \leq 100\%$) that expressed TRIM44 was evaluated for each case. Expression of TRIM44 was graded as high expression (intensity plus proportion scores ≥ 4 of tumor cells showing immunopositivity), or low expression (intensity plus proportion scores ≤ 3 of tumor cells showing immunopositivity) using high-power ($\times 200$) microscopy.[22]

Statistical analysis. Clinicopathological variables pertaining to the corresponding patients were analyzed for significance by chi-square-test or Fisher's exact test. For the analysis of survival, Kaplan–Meier survival curves were constructed for groups based on univariate predictors and differences between the groups were tested with the log–rank test. Univariate and multivariate survival analyses were carried out using the likelihood ratio test of the stratified Cox proportional hazards model. Differences were assessed with a two-sided test and considered significant at the $P < 0.05$ level.

Results

Protein expression of TRIM44 in gastric cancer cell lines. Western blot analysis was carried out using a TRIM44-specific antibody (Fig. 1A) to test what level of the TRIM44 protein is expressed in seven gastric cancer cells such as KatoIII, NUGC4, HGC27, MKN7, MKN28, MKN45, and MKN74. TRIM44 overexpression was observed in MKN28 and MKN45 cells (2/7 lines; 29%), suggesting this gene to be a target for activation in these cell lines. A formalin-fixed esophageal cancer TE13 cell line presenting overexpression of TRIM44, in which >50% of cells showed staining, was used as a positive control, whereas a formalin-fixed gastric cancer HGC27 cell line (data not shown) and TE13 staining without the TRIM44 antibody presented low expression of TRIM44 and was included as a negative control (Fig. S1).

Suppression of cell proliferation by downregulation of TRIM44 expression. To gain an insight into the potential role of *TRIM44* as an oncogene whose overexpression could be associated with gastric carcinogenesis, we first carried out a cell proliferation assay using siRNA specific to TRIM44 to investigate whether knockdown of TRIM44 expression would suppress proliferation of gastric cancer cells showing overexpression of the gene. In MKN28 and MKN45 (Fig. S2) cell lines, expression of the TRIM44 protein was more efficiently knocked down 24–96 h after the transient introduction of a TRIM44-specific siRNA (siRNA-TRIM44) than with the control siRNA (siRNA-control) (Fig. 1B). The proliferation of MKN28 cells was 22.5% lower than with controls after the knockdown of endogenous TRIM44 expression (Fig. 1C).

Suppression of cell migration and invasion by downregulation of TRIM44 expression. A Matrigel invasion assay was carried out to examine the invasive potential of MKN28 cells transfected with siRNA-TRIM44. The number of cells that migrated through the uncoated (migration assay) or Matrigel-coated (invasion assay) membrane into the lower chamber were significantly lower in siRNA-TRIM44 transfected cells than in siRNA-control transfected cells, suggesting that TRIM44 has invasive potential in gastric cancer cells (Fig. 1D).

Immunohistochemical analysis of TRIM44 expression in primary tumors of gastric cancer. As TRIM44 protein was overexpressed in some gastric cancer cell lines, it was hypothesized that TRIM44 was also highly expressed in gastric cancer tissues and assumed to be part of carcinogenesis and malignant outcomes. We examined the clinicopathological significance of TRIM44 expression in primary tumor samples of gastric cancer based on the immunohistochemical staining pattern of this protein. Specific immunostaining of the TRIM44 protein in primary samples was confirmed using cell lines as positive or negative controls (Figs 2A,S3). Expression of the TRIM44

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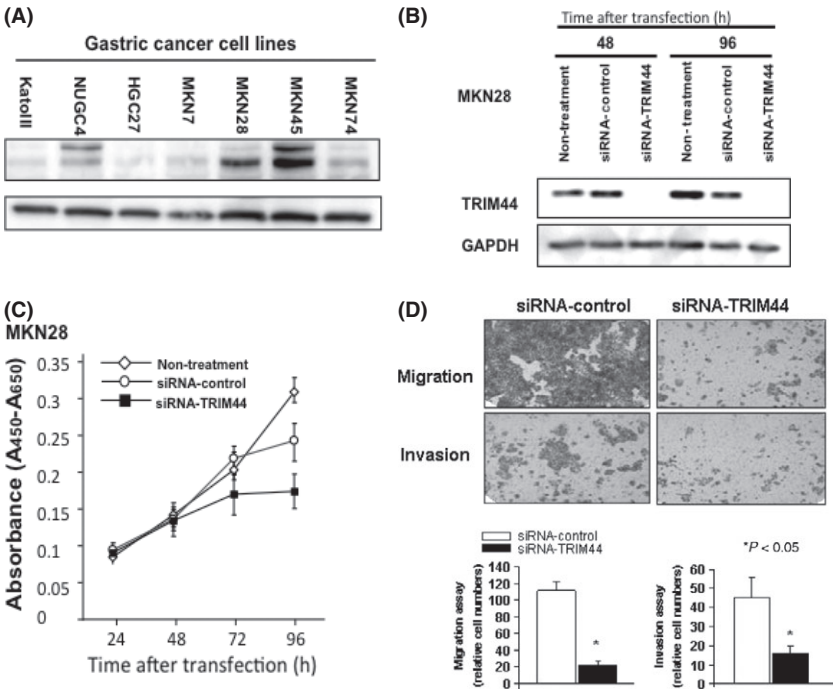

Fig. 1. (A) Western blot analysis using the tripartite motif-containing protein 44 (TRIM44)-specific antibody to test how much TRIM44 is expressed in seven gastric cancer cell lines. (B) Loss-of-function screening was done using siRNAs targeting TRIM44 in MKN28 cells. The knock-down of a target gene was confirmed by Western blotting. (C) For measurement of cell growth, the number of viable cells at various time points after transfection was assessed by colorimetric water-soluble tetrazolium salt assay. (D) Transwell migration and invasion assays.

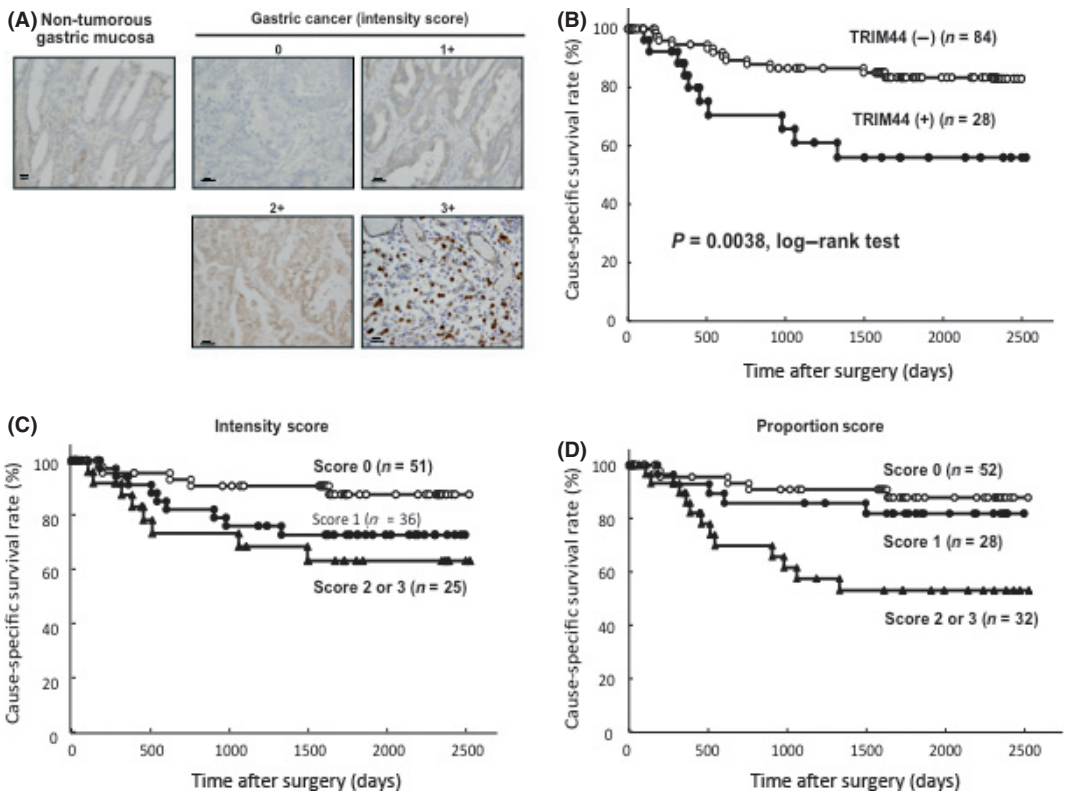

Fig. 2. (A) Specific immunostaining of tripartite motif-containing protein 44 (TRIM44) in primary samples was confirmed. Expression of the TRIM44 protein was observed in both the cytoplasm and nucleus of cancer cells. Intensity score: 0, negative; 1, weak; 2, moderate; 3, strong. Proportion score: 0 < 10%; 10% $\leq$ 1 $\leq$ 33%; 34% $\leq$ 2 $\leq$ 66%; 67% $\leq$ 3 $\leq$ 100%. (B) In the total scores of intensity plus proportion, the high expression group of TRIM44 presenting scores $\geq$4 of tumor cells showing immunopositivity, presented significantly poorer prognosis than the low expression group (P = 0.0038, log–rank test). Kaplan–Meier survival estimates showed that TRIM44 immunoreactivity in tumor cells was significantly associated with a worse overall survival according to the extent of intensity (C) and proportion (D).

protein was observed in both the cytoplasm and nucleus of cancer cells. We classified 112 gastric cancer tumors into positive and negative groups according to the intensity and proportion of TRIM44 staining among tumor cells. In primary cases, TRIM44 protein expression was negative in most of the non-tumorous gastric mucosal cell population (intensity score 0). Table S1 shows the distribution of patients with TRIM44 immunoreactivity in tumor cells according to the extent of intensity and proportion. Kaplan–Meier survival estimates showed that TRIM44 immunoreactivity in tumor cells was significantly associated with worse overall survival according to the extent of intensity and proportion (Fig. 2C,D). In the total scores of intensity plus proportion, the high expression group of TRIM44, with scores ≥ 4 of tumor cells showing immunopositivity, presented significantly poorer prognosis than the low expression group ($P = 0.0038$, log–rank test) (Fig. 2B). Five-year survival rates of patients with TRIM44 high expression versus low expression cancers in each stage were: 100% *vs* 98% ($P = 0.71$) in stage I; 50% *vs* 100% ($P = 0.09$) in stage II; 30% *vs* 36% ($P = 0.94$) in stage III; and 0% *vs* 33% ($P = 0.23$) in stage IV (figure not shown).

Association between TRIM44 protein levels and clinicopathological characteristics in primary cases of gastric cancer. The relationship between the expression of the TRIM44 protein and clinicopathological characteristics is summarized in Table 1. Protein expression of TRIM44 was significantly associated with an advanced type of macroscopic appearance, lymphatic invasion, and higher recurrence rate, and tended to be associated with depth of invasion in the TNM classification, whereas other characteristics including histological grade were not. Recurrences were evident in 24 (21%) of 112 patients. Twenty-two (92%) recurrent patients belonged to pathological stage II or more. Eleven (39%) patients had TRIM44 high expression cancer and 13 (15%) patients had TRIM44 low expression cancer (Table 1). Peritoneal recurrence was found more frequently in TRIM44 high expression cancer (TRIM44 high *vs* low, 82% [9/11] *vs* 38% [5/13]). In contrast, hematogenous recurrence was found more frequently in TRIM44 low expression cancer (TRIM44 high *vs* low, 0% [0/11] *vs* 46% [6/13]).

In the Cox proportional hazard regression model (Table 2), univariate analyses indicated that TRIM44 protein expression, location, macroscopic appearance, venous invasion, lymphatic invasion, pT category, and pN category were significantly associated with cause-specific survival. When data were stratified for multivariate analysis using both the forward and backward stepwise Cox regression procedures, TRIM44 immunoreactivity in tumor cells remained significant at $P < 0.05$ (hazard ratio, 3.37 [1.18–9.64]) for overall survival in all patients, suggesting that immunoreactivity can be an independent predictor of overall survival.

Table 1. Association between clinicopathologic characteristics and TRIM44 expression

	n	TRIM44 immunoreactivity		*P*-value*
		High expression, *n* (%)	Low expression, *n* (%)	
Total	112	28 (25)	84 (75)	
Gender				
Male	71	18 (64)	53 (63)	0.3025
Female	41	10 (36)	31 (37)	
Age, years				
Mean 61.75 (range, 27–87)				
<60	63	13 (46)	50 (60)	0.2263
≥60	49	15 (54)	34 (40)	
Location				
Upper	41	13 (46)	28 (33)	0.8648
Middle	58	11 (39)	47 (56)	
Lower	13	4 (14)	9 (11)	
Histopathological grading				
Differentiated	52	14 (50)	38 (45)	0.6617
Undifferentiated	60	14 (50)	46 (55)	
Macroscopic appearence				
Early	74	14 (50)	60 (71)	**0.0381**
Advanced	38	14 (50)	24 (29)	
Tumor size (mm)				
<30	49	11 (39)	38 (45)	0.5824
≥30	63	17 (61)	46 (55)	
Venous invasion				
0–1	103	26 (93)	77 (92)	**0.0003**
2–3	9	2 (7)	7 (8)	
Lymphatic invasion				
0–1	88	16 (57)	72 (86)	**0.0014**
2–3	24	12 (43)	12 (14)	
TNM classification				
pT categories				
pT1	67	11 (39)	56 (67)	0.0680
pT2	8	1 (4)	7 (8)	
pT3	16	7 (25)	9 (11)	
pT4	21	9 (32)	12 (14)	
pN categories				
N0	70	15 (54)	59 (70)	0.1830
N1	13	4 (14)	10 (12)	
N2	8	2 (7)	4 (5)	
N3	18	7 (25)	11 (13)	
pStage				
I	71	12 (43)	59 (70)	0.1395
II	12	4 (14)	8 (10)	
III	20	9 (32)	11 (13)	
IV	9	3 (11)	6 (7)	
Recurrence				
Absent	85	17 (61)	71 (85)	**0.0078**
Present	24	11 (39)	13 (15)	

Significant values are in boldface type. **P*-values are from chi-square-test or Fisher's exact test and were significant at <0.05.

Discussion

Ubiquitylation is one of the many post-translational modifications to regulate cellular physiology, and the ubiquitin-mediated proteolytic pathway has a pivotal role in the degradation of short-lived regulatory proteins, including those associated with cell cycle regulation, cellular signaling, DNA repair, morphogenesis, protein quality control, and transcriptional regulation. Most oncogene products and tumor suppressors are regulated by post-translational modifications, including the ubiquitin–proteasome system.[11]

Members of the family of TRIM-containing proteins could be defined as a subfamily of the RING type E3 ubiquitin ligase family[23] and contain more than 70 members in humans and mice. These TRIM family proteins are involved in a broad range of biological processes, including transcriptional regulation, cell growth, apoptosis, development, and tumorigenesis.[24,25] Several TRIM family genes were reported to positively or negatively regulate oncogenesis and tumor progression by affecting pathways such as cell proliferation, DNA repair, and apoptosis.[15] In gastric cancer, some proteins such as TRIM28,[26] TRIM29,[27] and TRIM31[28,29] were reported to have alterations in the TRIM proteins and genes associated with poor prognosis[26,27] and cell proliferation,[28,29] suggesting that other TRIM proteins associated with the progression of gastric cancer need to be identified.

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Table 2. Multivariate analysis using stepwise Cox regression procedures

Variables		Univariate†	Multivariate‡		
		P-value	HR	95% CI	*P*-value
Gender	Male *vs* female	0.4864		–	
Age, years	>60 *vs* <60	0.1170	5.917	1.748–20.00	**0.0043**
Location	U *vs* ML	**0.0192**		–	
Histological type	Undifferentiated *vs* differentiated	0.8503		–	
Macroscopic appearance	Types 1–4 *vs* type 0	**<0.0001**		–	
Tumor size (cm)	>3 *vs* <3	**<0.0001**		–	
Venous invasion	v2–3 *vs* v0–1	**<0.0001**		–	
Lymphatic invasion	ly2–3 *vs* ly0–1	**<0.0001**	2.333	1.324–4.110	**0.0034**
pT category	T3–4 *vs* T1–2	**<0.0001**	14.92	1.694–125.0	**0.0149**
pN category	N3 *vs* N0–2	**<0.0001**	8.780	2.746–28.06	**0.0002**
TRIM44 expression	High *vs* low	**0.0038**	3.371	1.179–9.637	**0.0233**

Significant values are in boldface type. †Kaplan–Meier method, and the significance was determined by log–rank test. ‡Multivariate survival analysis was carried out using Cox's proportional hazard model. CI, confidence interval; HR, hazard ratio; ML, middle and low; TRIM44, tripartite motif-containing protein 44; U, upper; —, no data.

A member of the TRIM protein family, TRIM44, has been cloned from mouse brain cDNA library.[16] It has been also identified as a clinically relevant prognostic marker for esophageal and junctional adenocarcinoma by gene expression array[18] and as an amplified gene in head and neck cancer.[17] These findings prompted us to determine the clinicopathological and prognostic significance of TRIM44 overexpression/activation in primary gastric cancer. However, to date, there has been no report on the clinical significance of TRIM44 in patients with primary gastric cancer. In the present study, we hypothesized that overexpression/activation of TRIM44 may promote tumor cell proliferation and/or survival in gastric cancer. To test this hypothesis, the expression status of TRIM44 and the clinicopathological as well as biological significance of its expression was examined in cell lines and primary tumors of gastric cancer. Consequently, we showed that TRIM44 was overexpressed in 25% (28/112) of primary gastric cancers as well as in 29% (2/7) of gastric cancer cell lines, and this overexpression was a predictor of poor prognosis independent of other prognostic factors. Both the intensity and proportion of TRIM44 activity were indicators of poor prognosis in gastric cancer patients. In addition, downregulation of TRIM44 expression suppressed cell proliferation, migration, and invasion in gastric cancer cell lines, although the detailed mechanisms of overexpression and cell proliferation of TRIM44 are under evaluation.

Other fascinating reports are that TRIM44 regulates ubiquitination and stabilizes the protein. The N-terminal region of TRIM44 contains a ZF UBP domain. This protein module is also found in the deubiquitinating enzymes containing ubiquitin-specific peptidase 33 (USP33)/the von Hippel–Lindau tumor suppressor protein-interacting deubiquitinating enzymes 1 (VDU1) and USP20/VDU2, members of the ubiquitin-specific protease family.[30,31] Previous reports have shown that USPs deubiquitinate and stabilize their substrates. Indeed, USP20/VDU2 was reported to deubiquitinate and stabilize its associated protein, HIF1α.[32] These data suggest that *TRIM44* may function as a "USP-like TRIM" and act as a cancer-promoting gene regulating deubiquitanation and stabilization of oncogenes. This mechanism is under evaluation.

In conclusion, this is the first report to show that TRIM44 has a crucial oncogenic role and is a potential therapeutic target in gastric cancer. We showed the frequent overexpression of the TRIM44 protein in gastric cancer and its prognostic value in these patients. Although studies of larger cohorts are needed to validate these findings before moving to a clinical setting, our results provide evidence that TRIM44 is an important molecular marker for determining malignant properties and is a target for molecular therapy in patients with this lethal disease.

Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Positive and negative control of tripartite motif-containing protein 44 (TRIM44) immunostaining using formalin-fixed esophageal TE13 cell line.

Fig. S2. Cell proliferation assay by downregulation of tripartite motif-containing protein 44 (TRIM44) expression in MKN45. Loss-of-function screening was done using siRNAs targeting TRIM44 in the MKN45 cell. The knockdown of a target gene was confirmed by Western blot analysis (left). For measurements of cell growth, the numbers of viable cells at various time points after transfection were assessed by the colorimetric water-soluble tetrazolium salt assay (right).

Fig. S3. Patterns of immunohistochemistry according to the differentiation of gastric cancer.

Table S1. Distribution of patients with tripartite motif-containing protein 44 (TRIM44) immunoreactivity in tumor cells according to the extent of intensity and proportion.

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