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A novel prognostic factor TRIM44 promotes cell proliferation and migration, and inhibits apoptosis in testicular germ cell tumor

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Key words

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Tripartite motif 44 (TRIM44) is one of the TRIM family proteins that are involved in ubiquitination and degradation of target proteins by modulating E3 ubiquitin ligases. TRIM44 overexpression has been observed in various cancers. However, its association with testicular germ cell tumor (TGCT) is unknown. We aimed to investigate the clinical significance of TRIM44 and its function in TGCT. High expression of TRIM44 was significantly associated with α feto-protein levels, clinical stage, nonseminomatous germ cell tumor (NSGCT), and cancer-specific survival ($P = 0.0009$, $P = 0.0035$, $P = 0.0004$, and $P = 0.0140$, respectively). Multivariate analysis showed that positive TRIM44 IR was an independent predictor of cancer-specific mortality ($P = 0.046$). Gain-of-function study revealed that overexpression of TRIM44 promoted cell proliferation and migration of NTERA2 and NEC8 cells. Knockdown of TRIM44 using siRNA promoted apoptosis and repressed cell proliferation and migration in these cells. Microarray analysis of NTERA2 cells revealed that tumor suppressor genes such as *CADM1*, *CDK19*, and *PRKACB* were upregulated in TRIM44-knockdown cells compared to control cells. In contrast, oncogenic genes including *C3AR1*, *ST3GAL5*, and *NT5E* were downregulated in those cells. These results suggest that high expression of TRIM44 is associated with poor prognosis and that TRIM44 plays significant role in cell proliferation, migration, and anti-apoptosis in TGCT.

Testicular germ cell tumors (TGCTs) represent 90–95% of testicular cancer, which is a relatively rare cancer that accounts for approximately 1–2% of male cancers.[1] TGCT is classified into seminomatous germ cell tumor (SGCT) or nonseminomatous germ cell tumor (NSGCT) depending on types of histological components. NSGCTs include yolk sac tumors, embryonal cell carcinomas, teratomas, and choriocarcinomas, with or without the presence of a seminomatous component. The cure rates for the good prognosis group is excellent with a 5-year overall survival of 86% and 92% for SGCT and NSGCT, respectively.[1] However, in the poor prognosis group, the disease could be lethal with a 5-year overall survival of 48% for NSGCT.

Tripartite motif (TRIM) protein family is known to contain the RING finger domain, which mainly functions as E3 ubiquitin ligases.[2] By functioning as E3 ubiquitin ligases, TRIM family proteins modulate ubiquitination leading to degradation, activation or functional modification of target proteins. Thus, they are involved in various biological processes including regulation of transcriptions, cell proliferation, apoptosis, and development.[3] Among the known 70 genes of the TRIM family, approximately 20 genes are associated with malignancies by regulating oncogenesis.[2–12]

TRIM44 was originally isolated from a mouse brain cDNA library.[13] TRIM44 is considered to be associated with several malignancies, such as lung cancer,[14] gastric cancer,[15] esophageal cancer,[16] and head/neck squamous cell carcinoma.[17] For instance, TRIM44 was upregulated in non-small cell lung cancer (NSCLC) tumors in a study analyzing 30 pairs of NSCLC tumors and the matched adjacent normal tissue.[14] Another study found that TRIM44 was significantly associated with higher recurrence rate and worse cancer-specific survival in patients with gastric cancer.[15] However, to our knowledge, there are no previous reports suggesting association between TRIM44 and TGCT.

In the present study, we investigated clinical significance of TGCT, and further carried out functional studies of TRIM44 using TGCT cell lines.

Materials and Methods

Patient characteristics and tissue preparation. One hundred and three testicular specimens were obtained from orchiectomies performed between 1985 and 2006. In 103 testicular specimens, 62 and 41 specimens were diagnosed as SGCT and NSGCT, respectively. Specimens that contained pure NSGCT

Table 1. Relationships between TRIM44 IR and clinical characteristics in TGCT (*n* = 103)

Clinical data	TRIM44 IR		
	Negative (*n* = 62)	Positive (*n* = 41)	*P* value
Age (years ± SD)	35.4 ± 10.8	31.9 ± 12.1	0.198
Tumor marker			
LDH (*n* = 98)			
Normal	31	17	0.5037
High	29	21	
Unknown	2	3	
AFP (*n* = 99)			
Normal	47	19	0.0009
High	12	21	
Unknown	3	1	
βhCG (*n* = 102)			
Normal	25	14	0.5892
High	37	26	
Unknown	0	1	
Tumor diameter (*n* = 92)			
≤10 cm	55	30	0.1006
>10 cm	2	5	
Unknown	5	6	
T stage			
T1	35	24	0.8341
≥T2	27	17	
N stage			
N0	50	22	0.0035
≥N1	12	19	
M stage			
M0	58	34	0.1093
M1	4	7	
S stage (*n* = 102)			
S0	17	7	0.2489
≥S1	45	33	
Unknown	0	1	
Stage (TNM classification)			
Stage 1	50	22	0.0035
≥Stage 2	12	19	
IGCCC risk group† (*n* = 31)			
Good	9	9	0.3142
Intermediate	2	7	
Poor	1	3	

†A total of 31 patients had metastasis (IGCCC can only be applied to cases associated with metastasis). AFP, α-fetoprotein; IGCCC, International Germ Cell Consensus Classification; IR, immunoreactivity; LDH, lactate dyhydrogenase; TGCT, testicular germ cell tumor;TRIM44, tripartite motif 44; βhCG, β human chorionic gonadotropin.

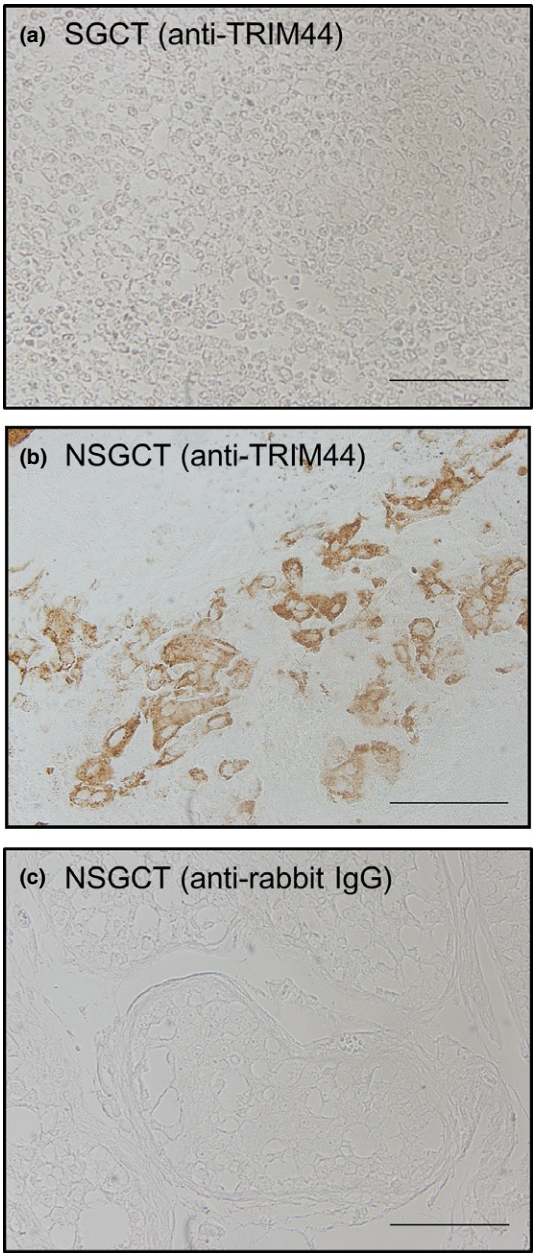

Fig. 1. TRIM44 was strongly expressed in nonseminomatous germ cell tumor (NSGCT). (a–c) Representative images of immunohistochemistry. (a) anti-TRIM44 in seminomatous germ cell tumor (SGCT), (b) anti-TRIM44 in nonseminomatous germ cell tumor (NSGCT), (c) negative control (NSGCT immunostained with rabbit IgG antibody). Scale bar = 100 μm.

component included seven embryonal carcinomas, one teratoma, one yolk sac tumor, and one choriocarcinoma. Thirty-one specimens showed a mixed component for NSGCT cases. Staging was performed according to the TNM 2009 staging system.[1] Patients with metastasis (31 cases) were also classified in terms of prognosis according to the International Germ Cell Consensus Classification (IGCCC).[18] No patient received chemotherapy or radiation before orchiectomy for TGCT. Of note, there were seven cancer-specific deaths, one of which had SGCT who developed late recurrence and died at 153 months after orchiectomy. At the time of recurrence, this patient already had multiple metastases including the liver, and had died of the disease before having been treated with chemotherapy.

This study was approved by our institutional ethical committee (#2283), and is in accordance with the Helsinki declaration. All the patients or their parents provided a written informed consent.

Immunostaining and immunohistochemical assessment. Sections were obtained from the same tumor blocks used for routine pathological evaluation. Therefore, hematoxylin and eosin (H&E) stained sections were also available for reference regarding areas of tumor and benign lesions.

Immunohistochemistry for TRIM44 expression was performed by the streptavidin-biotin method as previously

Table 2. Relationships between TRIM44 IR and pathological findings in TGCT (n = 103)

	TRIM44 immunoreactivity		
	Negative (n = 62)	Positive (n = 41)	P-value
Pathology			
SGCT	46	16	0.0004
NSGCT	16	25	
Tunica albuginea invasion			
Absent	44	29	0.4596
Present	18	11	
Unknown	0	1	
Venous invasion			
Absent	46	28	0.5145
Present	16	13	
Lymphatic vessel invasion			
Absent	54	31	0.1329
Present	8	10	
Tunica vaginalis invasion			
Absent	53	36	0.5603
Present	9	4	
Unknown	0	1	
Epididymis invasion			
Absent	53	36	0.5078
Present	7	5	
Unknown	2	0	
Spermatic cord invasion			
Absent	54	34	0.5570
Present	8	7	

IR was evaluated by using the intensity score. Intensity (0, none; 1, weak; 2, moderate; and 3, strong). Intensity score of 1 or over was defined as positive IR. Pearson's χ^2 test was used for statistical analysis except for 'Tunica vaginalis invasion', which was analyzed using a Fisher's test. IR, immunoreactivity; NSGCT, noseminomatous germ cell tumor; TGCT, testicular germ cell tumor; SGCT, seminomatous germ cell tumor; TRIM44, tripartite motif 44.

Table 3. Univariate and multivariate analyses with respect to cancer-specific survival in testicular germ cell tumor

Parameters	Univariate		Multivariate	
	OR (95% CI)	P-value	OR (95% CI)	P-value
S stage (S0 versus ≥S2)	3.2 (0.7–17.1)	0.144		
T stage (T1 versus ≥T2)	3.7 (0.7 – 26.4)	0.112		
N stage (N0 versus ≥N1)	17.0 (2.7–330.1)	0.001	7.9 (0.6–220.3)	0.127
M stage (M0 versus M1)	17.0 (3.2–102.2)	0.001	6.9 (0.9–74.0)	0.058
Vascular and/or lymphatic invasion (No versus Yes)	2.9 (0.6–15.7)	0.174		
Histology type (SGCT versus NSGCT)	4.2 (0.8–30.1)	0.079	0.4 (0–4.5)	0.417
TRIM44 IR (negative versus positive)	10.5 (1.7–201.7)	0.009	10.5 (1.0–299.0)	0.046

Logistic regression models were used for univariate and multivariate analysis. P-value of <0.05 was considered to be statistically significant. CI, confidence interval; IR, immunoreactivity; NSGCT, nonseminomatous germ cell tumor; OR, odds ratio; SGCT, seminomatous germ cell tumor; TRIM44, tripartite motif protein 44.

described.[19] For primary antibody, we applied 1:200 diluted rabbit polyclonal antibody for TRIM44 (constructed as described elsewhere).[20] Sections were washed in TBS after applying primary antibody overnight at 4°C, and then they were incubated with CSAII (Dako, Carpentaria, CA, USA). For negative controls, normal rabbit IgG was used instead of primary antibodies.

Immunostained slides were evaluated for intensity scores as described in previous literature.[19] IR was evaluated by intensity score of immunostaining, which was rated from 0 to 3+ (0: none, 1: weak, 2: moderate, 3: strong). "Positive IR" of TRIM44 protein was defined as having intensity score of 1+ and over. A score of 1+ was considered the cut-off point, since the average and median value of intensity score of TGCT was 0.51 and 0 in TGCT, respectively. The optimal cut-off value in receiver operating characteristic (ROC) curve analysis for predicting cancer-specific survival was also 1+ in TGCT patients. Two observers (YY and TF) evaluated the slides, and a third observer (JK) estimated the scores of the slides in case of disagreement between the two observers.

Antibodies. Anti-DYKDDDDK tag antibody (anti-flag antibody) was purchased from Wako Pure Chemical Industries (Osaka, Japan) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was purchased from Sigma-Aldrich Japan (Tokyo, Japan). Anti-TRIM44 polyclonal antibody was manufactured at our laboratory as described elsewhere in the previous report.[20] This antibody is an affinity purified rabbit polyclonal antibody raised by immunizing rabbits with a glutathione *S*-transferase (GST) fusion protein with amino acids of full-length mouse TRIM44 protein as an antigen. The antiserum was absorbed with GST-bound resin and anti-GST antibody was removed. The non-absorbed components were further purified by using an affinity column filled with the antigen. The quality and characterization of these antibodies were confirmed by Western blotting analysis of the human TRIM44-transfected 293 T cells.

Plasmid construction and transfection. N-terminally flag-tagged human TRIM44 cDNA was amplified by polymerase chain reaction (PCR) with specific primers. The generated amplicon was then subcloned into pCDNA3 (Invitrogen, St. Louis, MO, USA) to generate mammalian expression plasmid.

Cells were cultured in 6-well plates 24 h before transfection. Transfection of expression vectors containing flag-tagged human TRIM44 cDNA and expression vector alone was performed using Lipofectamine 3000 (Invitrogen), according to the manufacturer's protocol. The cell extracts were analyzed after 48 h by Western blotting.

Cell culture. 293T cells, and testis-originated NSGCT cells (NTERA2 and NEC8) were used in this study. All cell lines were cultured at 37°C in a humidified chamber with a 5% CO^2 atmosphere. Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute (RPMI) were purchased from Sigma-Aldrich Japan, and supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

Western blot analysis. Western blot analysis was performed as previously described.[21] 293T cells were plated in 100 mm culture dishes, and were transfected with 5 μg of expression vectors with TRIM44 recombinant plasmids (pCI-FLAG-TRIM44). These transfected 293T cells were used as positive

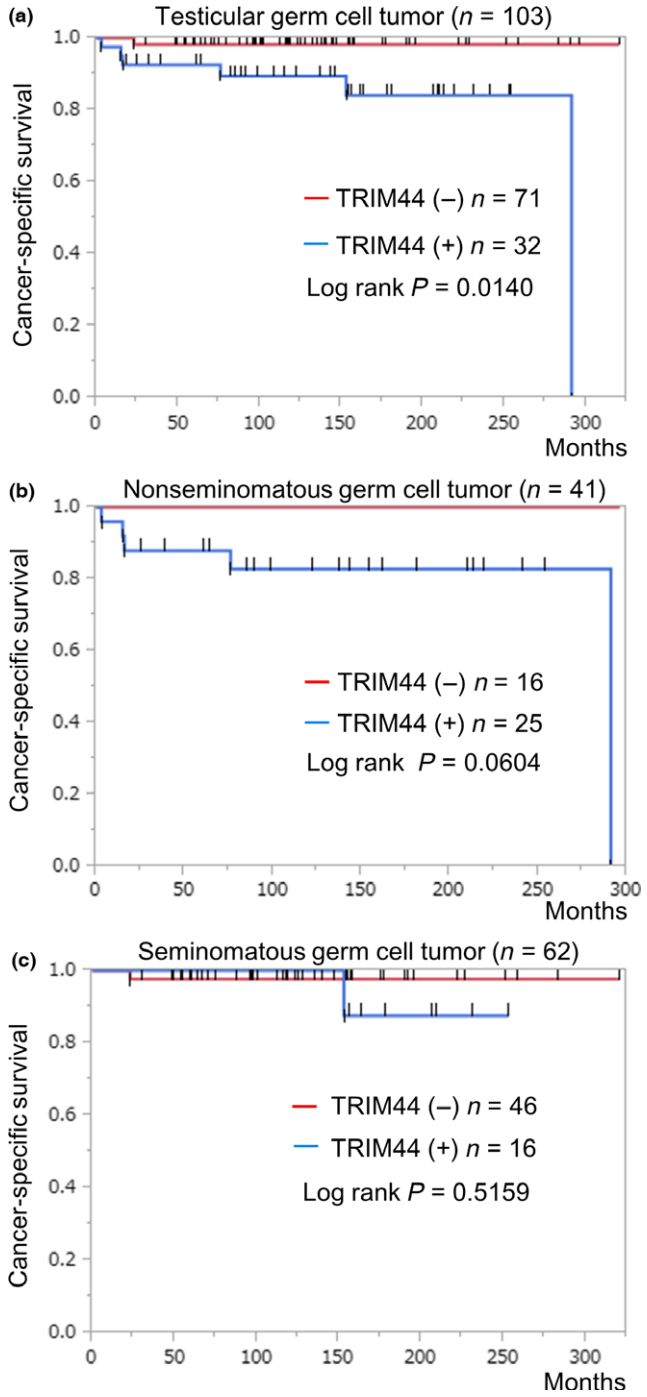

Fig. 2. Overexpression of TRIM44 was associated with cancer-specific survival in patients with testicular germ cell tumor (TGCT). (a) Cancer-specific survival of patients with TGCT according to TRIM44 immunoreactivity (IR); n = 103. Patients with positive TRIM44 IR showed worse prognosis (P = 0.0140, log-rank test). (b) There was a trend towards lower rate of cancer-specific survival in positive TRIM44 IR in patients with NSGCT (P = 0.0604, log-rank test). (c) There was no significant difference between positive and negative TRIM44 IR in terms of cancer-specific survival rate in SGCT patients (P = 0.5159, log-rank test).

control for TRIM44 expression. 293T cells transfected by empty vector (without tagged TRIM44) were used as negative control.

293T, NTERA2, and NEC8 cells were plated in 100 mm culture dishes. Cells were lysed with NP40 buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40) containing proteinase inhibitor. TGCT and 293T cell lysates were prepared in sodium dodecyl sulfate (SDS) sample buffer, and the proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE). Membranes were incubated with a primary antibody of anti-TRIM44, anti-flag, and anti-GAPDH antibody in a dilution of 1:500, 1:1000, 1:10 000, respectively. Bands were detected with either anti-rabbit IgG or anti-mouse IgG (GE Healthcare Japan K.K., Tokyo, Japan). Bands were then visualized with an enhanced chemiluminescence system (GE Healthcare Japan K.K., Tokyo, Japan).

Transfection efficiency of TRIM44 was extremely high in 293T cells compared to those with TGCT cells. Therefore, β-actin levels of 293T cells were adjusted separately from TGCT cells.

RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Total RNA extraction was performed using ISOGEN reagent (Nippon Gene, Tokyo, Japan). First-strand cDNA was generated by using PrimeScript (Takara, Kyoto, Japan). The resulting cDNA was subjected to real-time PCR using an Applied Biosystems 7300 real time PCR system based on SYBR Greenfluorescence (Thermo Fisher Scientific K.K., Kanagawa, Japan). mRNA expression levels were normalized by GAPDH. qRT-PCR was performed as previously described.[19] Sequences of PCR primers are described below.

GAPDH forward: 5′ – GGTGGTCTCCTCTGACTTCAACA
GAPDH reverse: 5′ – GTGGTCGTTGAGGGCAATG
TRIM44 forward: 5′ – GTGGACATCCAAGAGGCAAT
TRIM44 reverse: 5′ – AGCAAGCCTTCATGTGTCCT
CDK19 forward: 5′ – GAGCATGACTTGTGGCATATT
CDK19 reverse: 5′ – TGGATACCATCAAGAATCTGGT
CADM1 forward: 5′ – TAAAAGGCAAATCGGAGGTG
CADM1 reverse: 5′ – AGATCACTGGGACCCCATC
PRKACB forward: 5′ – TTTACCAGAGGAAGGTTGAAGC
PRKACB reverse: 5′ – GAGACACGGATATCTTCTTCTTCAT
C3AR1 forward: 5′ – ATGGCGTCTTTCTCTGCTG
C3AR1 reverse: 5′ – CCTGGCAATCCCAGTAAAAA
ST3GAL5 forward: 5′ – GAGCAATGCCAAGTGAGTACA
ST3GAL5 reverse: 5′ – GGGCCTTCTCATCTTGCTT
NT5E forward: 5′ – TGAATTATTAAGACATGACTCTGGTGA
NT5E reverse: 5′ – TGGAAAACTTGATCCGACCT

Small interfering RNA transfection. Downregulation of TRIM44 was carried out using small interfering RNA (siRNA) transfection. Three specific siRNAs targeting TRIM44, and one non-targeting siRNA (siRNA control) were purchased from Funakoshi (Tokyo, Japan). These siRNAs were transfected into TGCT cells (NTERA2 and NEC8) by using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Downregulation of TRIM44 was confirmed by qRT-PCR and Western blot analysis. siRNA sense sequences were

siControl: 5′ – GUACCGCACGUCAUUCGUAUC – 3′
siTRIM44 #1: 5′ – GAAUCAGUCGGAUACUCAUAG – 3′
siTRIM44 #2: 5′ – CCGAGUAAGCAGGGAUGUACU – 3′
siTRIM44 #3: 5′ – CCGCUAUGAUCGAAUUGGUGG – 3′

Cell proliferation assay. Cells were seeded in 96-well plates 24 h before transfection (4.0×10^3 cells/well for NTERA2 overexpression experiment and 3.0×10^3 cells/well for

others). MTS assay was carried out using The Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega KK, Osaka, Japan) according to the manufacturer's instructions at 24 and 48 h after transfection. Assays were performed in five wells and data are presented as mean value ± SD.

Cell migration assay. Migration assay was performed by using a cell culture insert with an 8.0 μm-pore sized polyethylene terephthalate (PET) filter (Becton Dickinson). DMEM medium without FBS was added to the lower chamber for NTERA2 cells. Similar procedure was carried out with NEC8 except by using RPMI instead of DMEM as medium. The cells on the upper surface of the filter were carefully removed 48 h after transfection and were wiped with a cotton swab. Then the filter was dipped in methanol for 30 min, washed with fresh PBS, and stained with Giemsa for 30 s. After three times of washing with fresh PBS, filters were mounted on glass slides. The cells migrated on the lower surface were counted in five randomly selected fields under a microscope at a magnification of ×200. Data are presented as mean value ± SD.

Cell apoptosis assay. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed using the DEADEND Fuorometric TUNEL System (Promega, Madison, WI, USA). Cells (1.0×10^5 per well) were seeded in 6-well culture plates and incubated for 24 h. Cells were transfected with siRNAs as described, and were replated to Poly-L-Lysine coated glass (Matsunami Glass Ind., Osaka, Japan) inside a 24-well culture plate. Forty-eight hours after transfection, cells were then treated with TUNEL staining according to the manufacturer's protocol. The slides were treated with 4′6′-diamidino-2-phenylindole dihydrochloride (DAPI) for nuclear staining. Signals were captured using digital microscope (VH-8000; Keyence, Osaka, Japan). Percentage of apoptotic cells were evaluated in five randomly selected fields (×100), and data are presented as mean value ± SD.

Microarray analysis. To identify genes regulated by TRIM44 in NTERA2 cells, NTERA2 cells were transfected with siTRIM44 or siControl. Total RNAs from NTERA2 transfected with siTRIM44 #3 or siControl were extracted by using Qiagen RNeasy Micro Kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's instructions. We confirmed that RNA integrity number (RIN) values were above 8.0 in all RNA samples. The GeneChip Human Exon 1.0 ST array (Affymetrix Japan, Tokyo, Japan) was used according to the manufacturer's protocol. Microarray procedure and data analysis were performed as previously described.[22] Fold changes of gene expressions were log2 transformed and cutoff values were set at 0.3 (upregulated) or –0.3 (downregulated).

Statistical analyses. We used the statistical software JMP Pro version 11.0.2 (SAS Institute Japan, Tokyo Japan.) for data analysis. Pearson's χ^2 test and Fisher's test were (used when frequency was <5) used for analysis of association between TRIM44 IR and clinicopathological parameters. Student's *t*-test was used in the analysis of qRT-PCR, MTS assay, TUNEL assay and migration assay. Log-rank test was performed to analyze the statistical difference of cancer-specific survival. Univariate and multiple logistic regression models were used

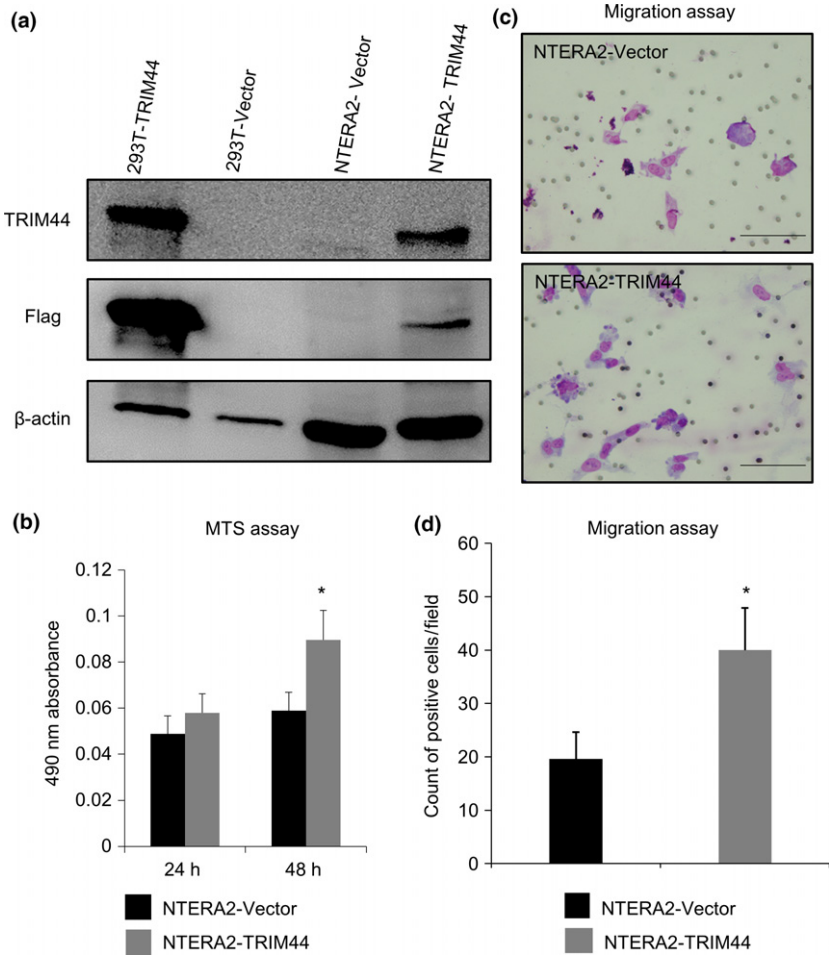

Fig. 3. Overexpression of TRIM44 promoted cell proliferation and migration in NTERA2 cells. (a) TRIM44 protein levels were analyzed in NTERA2 cells. 293T cells were transfected with TRIM44 DNA plasmid to obtain positive control for TRIM44. NTERA2 cells were transfected with pcDNA3-FLAG-TRIM44 or an empty vector. TRIM44 is overexpressed in NTERA2-TRIM44 cells. β-actin was used as loading control. (b) MTS assay of NTERA2-TRIM44 cells. NTERA2-TRIM44 cells promoted cell growth 48 h after transient transfection of TRIM44 (*$P < 0.05$). Results are presented as means and SD of five wells for NTERA2-Vector and NTERA2-TRIM44 cells. (c) Representative images of migration assay of NTERA-Vector and NTERA2-TRIM44 cells. Cell migration assay was performed in NTERA-Vector and NTERA2-TRIM44 cells. Average numbers of migrated cells were counted in five representative fields. (d) NTERA2-TRIM44 cells had higher motility (*$P < 0.05$; Student's *t*-test) than NETRA2-Vector cells. Migrated cells were counted in five randomly selected fields. Data are presented as mean value ± SD. Scale bar = 200 μm.

to evaluate independent predictors of cancer-specific mortality in TGCT patients. *P*-values < 0.05 were considered to be statistically significant.

Results

Clinical significance of TRIM44 IR in TGCT. Positive TRIM44 IR was significantly associated with elevated AFP level (normal versus high, $P = 0.0009$), N stage (N0 versus N1-3, $P = 0.0035$), and clinical stage (stage 1 versus ≥stage 2, $P = 0.0073$) (Table 1). Other tumor markers such as LDH and βhCG did not show significant association with positive TRIM44 IR.

Representative pictures of TRIM44 immunostaining in SGCT, NSGCT, and negative control are shown in Figure 1(a–c). SGCT and NSGCT showed positive IR for TRIM44 in 16/62 (25.8%) and 25/41 (86.8%) (Table 2). The rate of positive TRIM44 IR was significantly higher in patients with NSGCT compared with those of SGCT ($P = 0.0004$, Table 2). No significant findings were observed between TRIM44 IR and other pathological parameters other than histological types (Table 2).

We then investigated risk factors of cancer-specific mortality. N stage, M stage, and positive TRIM44 IR were significantly associated with cancer-specific mortality in the univariate analysis ($P = 0.001$, $P = 0.001$, and $P = 0.009$, respectively, Table 3). In the multivariate analysis, positive TRIM44 IR was an independent predictor of cancer-specific mortality in patients with TGCT ($P = 0.046$, Table 3).

Kaplan–Meier curves were evaluated for the difference between patients with negative and positive TRIM44 IR. Patients with positive TRIM44 IR had significantly higher mortality compared to those with negative TRIM44 IR in TGCT patients ($P = 0.0140$, Fig. 2a). NSGCT patients with positive TRIM44 had higher cancer-specific mortality than patients with negative TRIM44, although the difference was not significant ($P = 0.0604$, Fig. 2b). No difference was observed in SGCT patients ($P = 0.5159$, Fig. 2c).

TRIM44 overexpression promoted proliferation and migration of germ cell tumor cells. NTERA2 and NEC8 cells were transiently transfected with TRIM44 (Fig. 3a and Fig. S1a). TRIM44 transfected NTERA2 (Fig. 3b–d) and NEC8 (Fig. S1b–c) cells showed significantly higher cell proliferation and motility compared to control-transfected cells.

TRIM44 knockdown repressed cell proliferation and motility, and promoted cell apoptosis of TGCT cells. To assess the role of TRIM44 in TGCT, we performed loss-of-function study for TRIM44. Three TRIM44-specific siRNAs (siTRIM44 #1, siTRIM44 #2, siTRIM44 #3) were used for TRIM44 knockdown (Fig. 4a,b and Fig. S2a,b, respectively). Significant

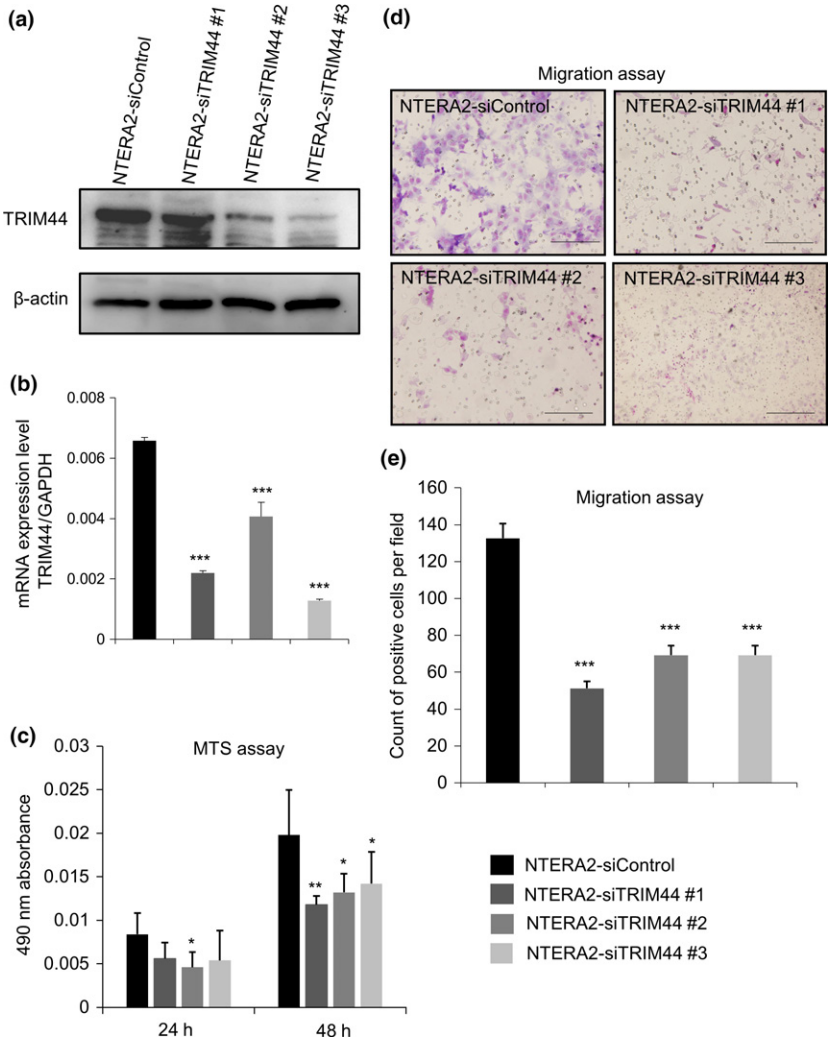

Fig. 4. Inhibition of TRIM44 showed an anticancer effect *in vitro* in NTERA2 cells. Three TRIM44-specific siRNAs (siTRIM44 #1, siTRIM44 #2, siTRIM44 #3) were used for TRIM44 knockdown. (a) TRIM44 protein levels were analyzed by Western blot analysis. NTERA2 cells treated with siControl showed expression of TRIM44 protein, whereas NTERA2-siTRIM44 cells showed reduced expression levels of TRIM44 protein. (b) Measurement of TRIM44 mRNA levels of NTERA2-siControl and NTERA2-siTRIM44 cells was performed. The results are presented as the average of three wells ± SD (***$P < 0.0001$). (c) Cell proliferation was measured by MTS assay at the indicated time points after siTRIM44 or siControl transfection (*$P < 0.05$, **$P < 0.005$, Student's *t*-test). Results are presented as means and SD of five wells for NTERA2-siControl and each type of NTERA2-siTRIM44 cells. (d) Representative images of cell migration assay of NTERA2-siControl and NTERA2-siTRIM44 cells. Scale bar = 200 μm. (e) Migrated cells of NTERA2-siControl and NTERA2-siTRIM44 were counted in five randomly selected fields. NTERA2-siTRIM44 cells had significantly lower motility compared to that of NTERA2-siControl (***$P < 0.0001$). Data are presented as mean value ± SD.

suppression of cell proliferation was observed after 48 h in NTERA2 and NEC8 cells (Fig. 4c and Fig. S2c).

Next, cell migration assay was carried out to determine the motility of TGCT cells. The number of migrated cells was significantly decreased in siTRIM44-treated cells than in siControl-treated cells (Fig. 4d,e and Fig. S3a,b).

In addition, TUNEL assay was performed to assess the role of TRIM44 on apoptosis in TGCT cells. TRIM44 knockdown using siRNAs increased apoptosis in NTERA2 and NEC8 cells (Fig. 5 and Fig. S4).

TRIM44 knockdown upregulates apoptosis-related genes and downregulates oncogenic genes. We conducted a microarray analysis to investigate genes that were potentially regulated by TRIM44 by identifying differentially expressed genes in NTERA2 cells treated with TRIM44 knockdown. Top 20 TRIM44 upregulated and downregulated genes were listed in Table 4. Oncogenic genes were highlighted in dark gray color and tumor suppressive genes were in light gray. Nine out of top 20 downregulated genes were oncogenes, and 8 out of 20 top upregulated genes were tumor suppressive genes (Table 4).

We then chose six candidate genes from these oncogenic and tumor suppressive genes as candidates for evaluating mRNA levels by qRT-PCR, since these genes were well-known cancer-related genes (Fig. 6a–f).

Together with the results of gain-of-function and loss-of-function experiments, TRIM44 is involved in anti-apoptosis and may directly or indirectly affect cell proliferation and migration via regulating tumorigenesis-related genes (Fig. 6g).

Discussion

The present study shows that TRIM44 expression is an independent significant prognostic factor of cancer-specific survival in patients with TGCT. TRIM44 overexpression was markedly correlated with NGCT, which may lead to the difference in malignant outcome. *In vitro* studies revealed that overexpression of TRIM44 promoted cell proliferation and motility. In addition, we also found that TRIM44 knockdown induces apoptosis and represses cell proliferation and migration of TGCT cells. Our findings suggest that TRIM44 plays an important role in the growth, migration and anti-apoptosis in TGCT.

The mechanism of TRIM44 related tumorigenesis is not well known. Ong *et al.*[16] have investigated the TRIM44 gene by gene expression arrays, and found that TRIM44 overexpression was associated in 15.9% of esophageal cancers, 19.8% of breast cancers, and 16.1% of all epithelial cancers. TRIM44 knockdown of gastric cancer cells using a small interfering RNA caused a decreased enrichment in the mTOR signature compared with cells treated with control siRNA.[16] In another study, TRIM44 promoted invasion and migration of NSCLC cells through activating NF-κB signaling pathway.[14] In the present study, we performed microarray analysis to explore TRIM44-regulated genes. In contrast, neither of these reported pathways was associated with TRIM44. Interestingly, microarray analysis showed that nine out of top 20 downregulated genes had oncogenic function (*C3AR1*,[23] *FMN1*,[24] *GBP1*,[25] *ST3GAL5*,[26] *NT5E*,[27–32] *RAB27B*,[33] *FBP2*,[34] *HIPK3*,[35] *PLAU*,[36] and 8 out of 20 top up-regulated genes had tumor suppressive function (*NUPR1*,[37,38] *CDK19*,[39] *CADM1*,[40–45] *INHBA*,[46] *TNFSF10*,[47] *PRKACB*,[48] *PCDHB6*,[49] *DDIT4*[50]). Furthermore, six of these eight tumor suppressive genes are associated with apoptotic mechanisms (*NUPR1*,[38] *CDK19*,[39] *CADM1*,[43,45] *INHBA*,[46] *TNFSF10*,[47] *DDIT4*[50]). This finding is in line with the present results of the apoptosis assay, that TRIM44-knockdown promoted apoptosis in TGCT cells.

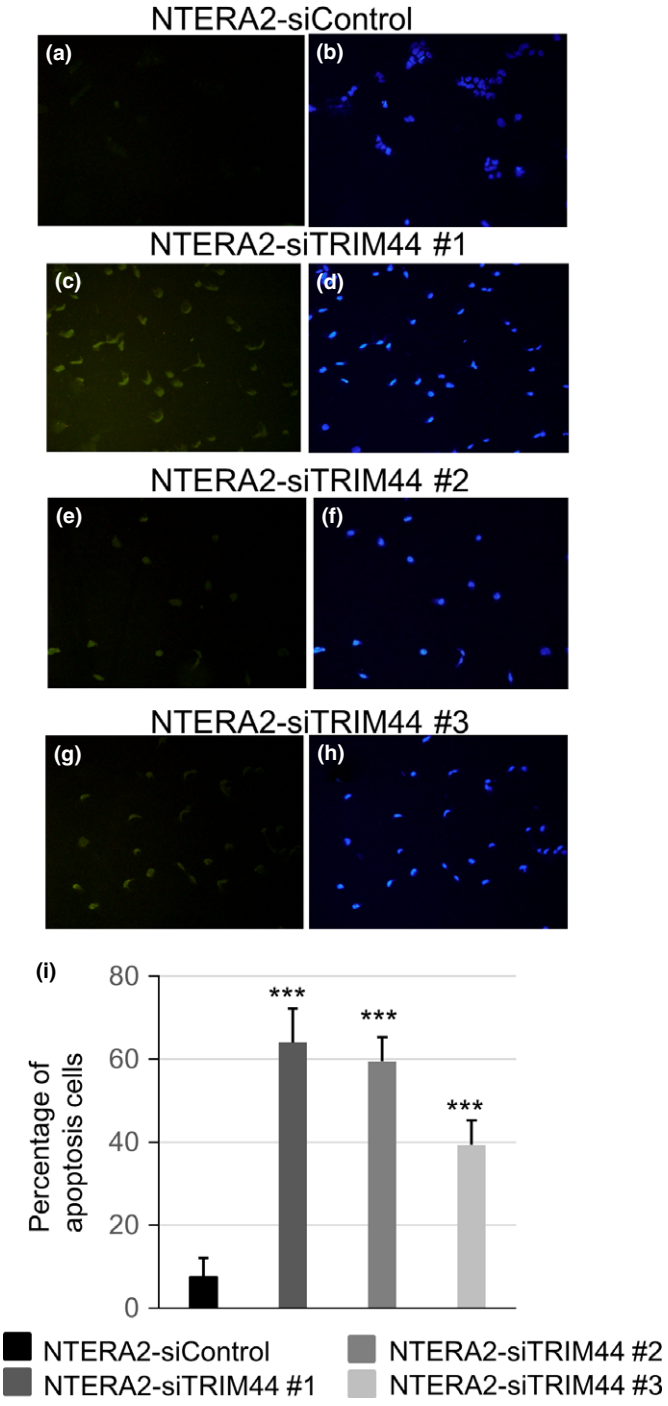

Fig. 5. Inhibition of TRIM44 promoted apoptosis in NTERA2 cells. TUNEL (TdT-mediated dUTP Nick-End Labeling) assay was performed to investigate apoptosis in TRIM44 knockdown NTERA2 (NTERA2-siTRIM44) and NTERA2-siControl cells. Cells showing blue light (stained with DAPI) were counted as total number of cells, and cells showing green light (stained with TdT) were counted as apoptotic cells. Percentage of apoptotic cells to total number of cells were calculated in five randomly selected microfields (×100). Significantly more apoptotic cells were observed in NTERA2-siTRIM44 cells than in NTERA2-siControl cells (***$P < 0.0001$, versus control, Student's *t*-test).

Among the top TRIM44-regulated genes that are presented from our microarray data, *NT5E* is a unique oncogenic gene

Table 4. Genes involved in TRIM44 knockdown NTERA2 cells (Top 20 regulated genes)

Upregulated			Downregulated		
Gene symbol	Description	Fold change	Gene symbol	Description	Fold change
ZNF487P	Regulation of transcription	2.55	*TRIM44*		0.42
IL2ORB	Blood coagulation	2.19	*C3AR1*	Melanoma tumorigenesis[23]	0.42
TMEM178	Integral to membrane	2.02	*FMN1*	Cell proliferation[24]	0.44
NUPR1	Reduces tumor growth in PCa[37,38]	1.97	*CDRT1*	Biological process	0.53
DDR2	Regulation of cell growth	1.86	*IFIT1*	Inhibits viral replication	0.54
ALDH1L2	One-carbon metabolic process	1.83	*GBP1*	Glioma cell proliferation[25]	0.55
CDK19	Cyclin-dependent protein kinase activity[39]	1.81	*PIG-S*	Attachment of GPI anchor to protein	0.56
CADM1	Apoptosis, cell adhesion[40–45]	1.80	*UGT2B7*	Lipid metabolic process	0.56
INHBA	Cell cycle arrest[46]	1.72	*GBP3*	Nucleotide binding	0.57
ITGA11	Cell migration	1.71	*EFCAB4B*	Ca(2+)-binding protein	0.57
SLC7A11	Amino acid transport	1.70	*KIR2DL3*	Immune response	0.57
TNFSF10	Induction of apoptosis[47]	1.70	*ST3GAL5*	Cell proliferation[26]	0.57
ZSCAN5B	Regulation of transcription	1.68	*NT5E*	Cancer progression[27–32]	0.58
PRKACB	Protein phosphorylation, inhibits cell proliferation[48]	1.67	*IGKC*	Prognostic marker in breast cancer	0.59
CMPK1	Nucleobase	1.65	*RAB27B*	Member of RAS oncogene[33]	0.59
B3GALT5	Protein glycosylation	1.65	*RNF185*	Protein binding	0.59
COL11A2	Skeletal system development	1.64	*FBP2*	Tumor growth in HCC[34]	0.61
TAGAP	Signal transduction	1.64	*HIPK3*	Anti-apoptosis[35]	0.62
PCDHB6	Cell adhesion[49]	1.62	*PLAU*	Cell migration[36]	0.62
DDIT4	Apoptosis, inhibits mTORC1[50]	1.61	*ZNRF4*	Protein degradation	0.62

Differentially expressed genes were identified by microarray analysis in TRIM44 knockdown NTERA cells. Top 20 regulated genes are presented for upregulation and downregulation. Genes highlighted with light gray are tumor suppressive genes, while genes with dark gray are oncogenic genes. siTRIM44 #3 was used for TRIM44 knockdown in NTERA2 cells. HCC, hepatocellular carcinoma; PCa, prostate cancer; TRIM, tripartite motif.

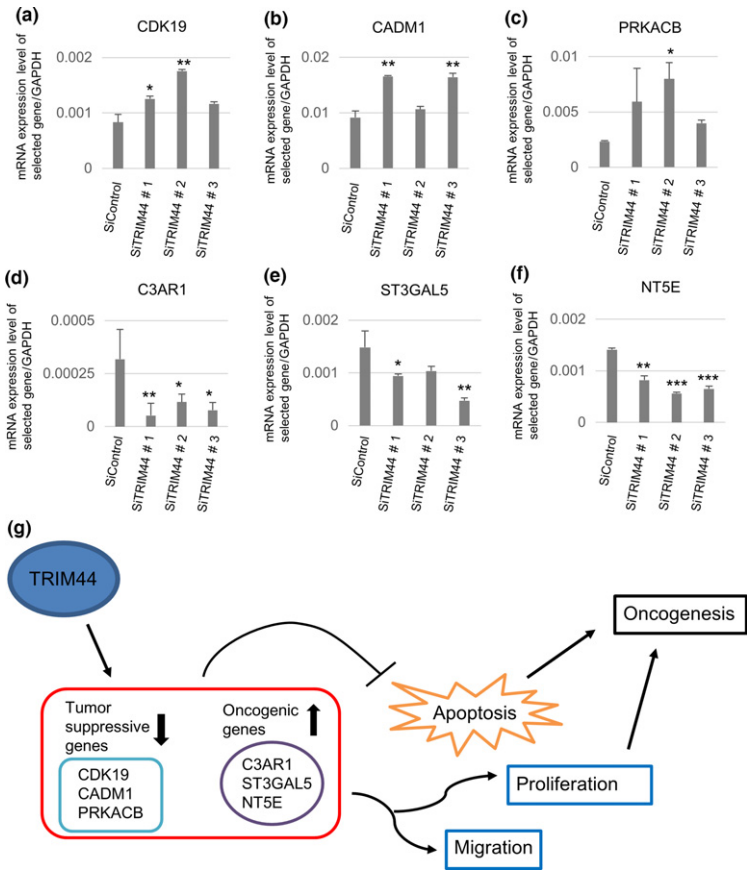

Fig. 6. mRNA levels of oncogenic and tumor suppressive genes regulated by siTRIM44 in NTERA2 cells and a proposed model of TRIM44 action in TGCT. (a–f): qRT- PCR was used for measuring mRNA levels of the six tumorigenesis-related candidate genes that were highly regulated by siTRIM44. mRNA levels of these tumorigenesis-related candidate genes were in line with the microarray results. *P*-values are presented versus control; $*P < 0.05$, $**P < 0.005$, $***P < 0.0001$, Student's *t*-test. (g): Schematic figure of proposed mechanism of TRIM44 involvement in tumorigenesis. TRIM44 may promote cell proliferation, migration, and inhibit apoptosis by regulating oncogenic (*C3AR1, ST3GAL5, NT5E*) and tumor suppressive (*CDK19, CADM1, PRKACB*) genes.

that is involved in ATP metabolism. This purine salvage enzyme generates adenosine from ATP/AMP.[29] Since adenosine binds to purine receptors and inhibits cell-mediated immune responses to tumor cells, *NT5E* is considered to have a key role in promoting cancer cell proliferation, migration, and tumor immune escape.[29] Clinical significance of NT5E overexpression has also been observed in various types of cancers including gastric cancer,[30] colorectal cancer,[31] and prostate cancer.[32] Together with our findings, TRIM44 may promote tumorigenesis via upregulation of NT5E.

CADM1 was also one of the top regulated genes by TRIM44-knockdown in a microarray analysis. *CADM1* is a well-known tumor suppressor gene that encodes an immunoglobulin superfamily cell adhesion molecule.[41,42] It involves intercellular adhesion via calcium-independent homophilic cell–cell interaction.[41] Therefore, loss of CADM1 function may enable cancer cells to metastasize. In fact, expression of CADM1 is commonly down regulated in many types of cancer, and is associated with cancer cell invasion, migration, and poor prognosis.[40–45] Since our microarray results showed that CADM1 was overexpressed in TRIM44-knockdown NTERA2 cells, TRIM44 may promote cancer migration through suppressing CADM1.

There are several concerns to this study. First of all, NSGCT patients with positive TRIM44 IR had higher cancer-specific mortality than patients with negative TRIM44 IR. However, the difference was not significant, probably due to the limited number of cases.

Secondly, there were concerns regarding the effect of siTRIM44 #1 on the biological phenomena of cancer cells in our experiment. Although the protein level of TRIM44 was most strongly downregulated by siTRIM44 #3, cell proliferation and motility were most strongly affected by siTRIM44. One possible explanation was that there might be some off-target effects of siTRIM44 #1 on cell function. Nevertheless, the differences between the effect of siTRIM44 #1 and #3 were not statistically significant in the cell proliferation and migration assay. Moreover, we observed similar results by using three siRNAs for these experiments, indicating the reliability of these results.

Of note, in some genes such as CDK19 and PRKACB, mRNA levels were not statistically significant in siTRIM44 #3 treated NTERA2 cells. However, the changes in mRNA levels showed similar changes that were in line with the microarray results, and moreover we observed similar results by using three siRNAs for these experiments, indicating the reliability of these results.

Finally, we have yet to evaluate the molecular regulation by TRIM44 in a protein level. It is notable that TRIM44 is involved in ubiquitination,[3] and the substrates involved in this pathway are not clear. Thus, we assume that it is necessary in the future studies to show these substrates at the protein level.

In conclusion, our study suggests that TRIM44 represses apoptosis and promotes cell proliferation and migration, leading to poor prognosis in patients with TGCT via regulating cancer-related genes such as *NT5E* and *CADM1*. These findings may shed new light to a new prognostic marker for TGCTs.

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Disclosure Statement

The authors have no conflict of interest.

Abbreviations

AFP	α feto-protein
IGCCC	International Germ Cell Consensus Classification
IR	immunoreactivity
LDH	lactate dehydrogenase
NSGCT	nonseminomatous germ cell tumor
qRT-PCR	quantitative reverse transcription polymerase chain reaction
SGCT	seminomatous germ cell tumor
TGCT	testicular germ cell tumor
TRIM	Tripartite motif
βhCG	β human chorionic gonadotropin

References

1 Albers P, Albrecht W, Algaba F *et al.* European Association of Urology. Guidelines on testicular cancer. [Cited 13 June 2015.] Available from URL: http://uroweb.org/guideline/testicular-cancer.
2 Hatakeyama S. TRIM proteins and cancer. *Nat Rev* 2011; **11**: 792–804.
3 Ikeda K, Inoue S. TRIM proteins as RING finger E3 ubiquitin ligases. *Adv Exp Med Biol* 2012; **770**: 27–37.
4 Liu C, Huang X, Hou S, Hu B, Li H. Silencing of tripartite motif (TRIM) 29 inhibits proliferation and invasion and increases chemosensitivity to cisplatin in human squamous cancer NCI-H520 cells. *Thorac Cancer* 2015; **6** (1): 31–7.
5 Wang Y, He D, Yang L *et al.* TRIM26 functions as a novel tumor suppressor of hepatocellular carcinoma and its down regulation contributes to worse prognosis. *Biochem Biophys Res Commun* 2015; **463**: 458–65.
6 Qiu F, Xiong JP, Deng J, Xiang XJ. TRIM29 functions as an oncogene in gastric cancer and is regulated by miR-185. *Int J Clin Exp Pathol* 2015; **8**: 5053–61.
7 Kosaka Y, Inoue H, Ohmachi T *et al.* Tripartite motif-containing 29 (TRIM29) is a novel marker for lymph node metastasis in gastric cancer. *Ann Surg Oncol* 2007; **14**: 2543–9.
8 Suzuki T, Urano T, Tsukui T *et al.* Estrogen-responsive finger protein as a new potential biomarker for breast cancer. *Clin Cancer Res* 2005; **11**: 6148–54.
9 Urano T, Saito T, Tsukui T *et al.* Efp targets 14-3-3 sigma for proteolysis and promotes breast tumour growth. *Nature* 2002; **417**(6891): 871–5.
10 Fujimura T, Inoue S, Urano T *et al.* Increased expression of Tripartite motif (TRIM) 47 is a negative prognostic predictor in human prostate cancer. *Clin Genitourin Cancer* 2016; **14**(4): 298–303.
11 Liang J, Xing D, Li Z, Shen J, Zhao H, Li S. TRIM59 is upregulated and promotes cell proliferation and migration in human osteosarcoma. *Mol Med Rep* 2016; **13**: 5200–6.
12 Fujimura T, Takahashi S, Urano T *et al.* Expression of androgen and estrogen signaling components and stem cell markers to predict cancer progression and cancer-specific survival in patients with metastatic prostate cancer. *Clin Cancer Res* 2014; **20**: 4625–35.
13 Botou E, Matsas R, Mamalaki A. Isolation of a mouse brain cDNA expressed in developing neuroblasts and mature neurons. *Brain Res Mol Brain Res* 2001; **86**(1–2): 153–67.
14 Luo Q, Lin H, Ye X, Huang J, Lu S, Xu L. Trim44 facilitates the migration and invasion of human lung cancer cells via the NF-κβ signaling pathway. *Int J Clin Oncol* 2015; **20**: 508–17.
15 Kashimoto K, Komatsu S, Ichikawa D *et al.* Overexpression of TRIM44 contributes to malignant outcome in gastric carcinoma. *Cancer Sci* 2012; **103**: 2021–6.
16 Ong CA, Shannon NB, Ross-Innes CS *et al.* Amplification of TRIM44: pairing a prognostic target with potential therapeutic strategy. *J Natl Cancer Inst* 2014; **106**(5): dju050. doi: 10.1093/jnci/dju050.

17 Järvinen AK, Autio R, Kilpinen S *et al.* High-resolution copy number and gene expression microarray analyses of head and neck squamous cell carcinoma cell lines of tongue and larynx. *Genes Chromosom Cancer* 2008; **47**: 500–9.
18 International germ cell consensus classification: a prognostic factor-based staging system for metastatic germ cell cancers. International Germ Cell Cancer Collaborative Group. *J Clin Oncol* 1997; **15**: 594–603.
19 Yamada Y, Fujimura T, Takahashi S *et al.* Clinical significance of amyloid precursor protein in patients with testicular germ cell tumor. *Adv Urol* 2013; 2013, Article ID 348438, 6 pages, doi:10.1155/2013/348438.
20 Orimo A, Inoue S, Ikeda K, Noji S, Muramatsu M. Molecular cloning, structure and expression of mouse estrogen-responsive finger protein Efp. Co-localization with estrogen receptor mRNA in target organs. *J Biol Chem* 1995; **270**: 24406–13.
21 Takayama K, Kaneshiro K, Tsutsumi S *et al.* Identification of novel androgen response genes in prostate cancer cells by coupling chromatin immunoprecipitation and genomic microarray analysis. *Oncogene* 2007; **26**: 4453–63.
22 Takayama K, Suzuki T, Tsutsumi S *et al.* Integrative analysis of FOXP1 function reveals a tumor-suppressive effect in prostate cancer. *Mol Endocrinol* 2014; **28**: 2012–24.
23 Nabizadeh JA, Manthey HD, Steyn FJ *et al.* The component C3a receptor contributes to melanoma tumorigenesis by inhibiting neutrophil and CD4+ cell responses. *J Immunol* 2016; **196**: 4783–92.
24 Hu J, Lu J, Lian G, Ferland RJ, Dettenhofer M, Sheen VL. Formin 1 and filamin B physically interact to coordinate chondrocyte proliferation and differentiation in the growth plate. *Hum Mol Genet* 2014; **23**: 4663–73.
25 Li L, Ma G, Jing C, Liu Z. Guanylate-binding protein 1 (GBP1) promotes lymph node metastasis in human esophageal squamous cell carcinoma. *Discov Med* 2015; **20**(112): 369–78.
26 Liang YJ, Ding Y, Levery SB, Lobaton M, Handa K, Hakomori SI. Differential expression profiles of glycosphingolipids in human breast cancer stem cells vs. cancer non-stem cells. *Proc Natl Acad Sci USA* 2013; **110**: 4968–73.
27 Turcotte M, Spring K, Pommey S *et al.* CD73 is associated with poor prognosis in high-grade serous ovarian cancer. *Cancer Res* 2015; **75**: 4494–503.
28 Zhang B, Song B, Wang X *et al.* The expression and clinical significance of CD73 molecule in human rectal adenocarcinoma. *Tumour Biol* 2015; **36**: 5459–66.
29 Gao ZW, Dong K, Zhang HZ. The roles of CD73 in cancer. *Biomed Res Int* 2014; 2014, Article ID 460654, 9 pages, doi:10.1155/2014/460654.
30 Lu XX, Chen YT, Feng B, Mao XB, Yu B, Chu XY. Expression and clinical significance of CD73 and hypoxia-inducible factor-1α in gastric carcinoma. *World J Gastroenterol* 2013; **19**: 1912–8.
31 Wu X, He XS, Chen YF *et al.* High expression of CD73 as a poor prognostic biomarker in human colorectal cancer. *J Surg Oncol* 2012; **106**(2): 1307. doi: 10.1002/jso.23056.
32 Yang Q, Du J, Zu L. Overexpression of CD73 in prostate cancer is associated with lymph node metastasis. *Pathol Oncol Res* 2013; **19**: 811–4.
33 Zhao H, Wang Q, Wang X *et al.* Correlation between RAB27B and p53 expression and overall survival in pancreatic cancer. *Pancreas* 2016; **45**: 204–10.
34 Samarin J, Laketa V, Malz M *et al.* PI3K/AKT/mTOR-dependent stabilization of oncogenic far-upstream element proteins in hepatocellular carcinoma cells. *Hepatology* 2016; **63**: 813–26.
35 Curtin JF, Cotter TG. JNK regulates HIPK3 expression and promotes resistance to Fas-mediated apoptosis in DU 145 prostate carcinoma cells. *J Biol Chem* 2004; **279**: 17090–100.
36 Banyard J, Chung I, Migliozzi M *et al.* Identification of genes regulating migration and invasion using a new model of metastatic prostate cancer. *BMC Cancer* 2014; **14**: 387.
37 Jiang WG, Davies G, Martin TA, Kynaston H, Mason MD, Fostad O. Com-1/p8 acts as a putative tumour suppressor in prostate cancer. *Int J Mol Med* 2006; **18**: 981–6.
38 Malicet C, Lesavre N, Vasseur S, Iovanna JL. p8 inhibits the growth of human pancreatic cancer cells and its expression is induced through pathways involved in growth inhibition and repressed by factors promoting cell growth. *Mol Cancer* 2003; **2**: 37.
39 Trakala M, Malumbres M. Cyclin C surprises in tumour suppression. *Nat Cell Biol* 2014; **16**: 1031–3.
40 Wikman H, Westphal L, Schmid F *et al.* Loss of CADM1 expression is associated with poor prognosis and brain metastasis in breast cancer patients. *Oncotarget* 2014; **5**: 3076–87.
41 Masuda M, Yageta M, Fukuhara H *et al.* The tumor suppressor protein TSLC1 is involved in cell-cell adhesion. *J Biol Chem* 2002; **277**: 31014–9.
42 Kuramochi M, Fukuhara H, Nobukuni T *et al.* TSLC1 is a tumor-suppressor gene in human non-small-cell lung cancer. *Nat Genet* 2001; **27**: 427–30.
43 Lu B, Di W, Wang H, Ma H, Li J, Zhang Q. Tumor suppressor TSLC1 is implicated in cell proliferation, invasion and apoptosis in laryngeal squamous carcinoma by regulating Akt signaling pathway. *Tumour Biol* 2012; **33**: 2007–17.
44 Takahashi Y, Iwai M, Kawai T *et al.* Aberrant expression of tumor suppressors CADM1 and 4.1B in invasive lesions of primary breast cancer. *Breast Cancer* 2012; **19**: 242–52.
45 Lung HL, Cheung AK, Xie D *et al.* TSLC1 is a tumor suppressor gene associated with metastasis in nasopharyngeal carcinoma. *Cancer Res* 2006; **66**: 9385–92.
46 Kim YI, Kim BH, Khang I, Cho BN, Lee HK. Cell growth regulation through apoptosis by activin in human gastric cancer SNU-16 cell lines. *Oncol Rep* 2009; **21**: 491–7.
47 Johnstone RW, Frew AJ, Smyth MJ. The TRAIL apoptotic pathway in caner onset, progression and therapy. *Nat Rev Cancer* 2008; **8**: 782–98.
48 Chen Y, Gao Y, Tian Y, Tian DL. PRKACB is downregulated in non-small cell lung cancer and exogeneous PRKACB inhibits proliferation and invasion of LTEP-A2 cells. *Oncol Lett* 2013; **5**: 1803–8.
49 Guo H, Nairn A, dela Rosa M *et al.* Transcriptional regulation of the protocadherin β cluster during Her-2 protein-induced mammary tumorigenesis results from altered N-glycan branching. *J Biol Chem* 2012; **287**: 24941–54.
50 DeYoung MP, Horak P, Sofer A, Sgroi D, Ellisen LW. Hypoxia regulates TSC1/2-mTOR signaling and tumor suppression through REDD1-mediated 14-3-3 shuttling. *Genes Dev* 2008; **22**: 239–51.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. MTS assay and migration assay of TRIM44-overexpressed NEC8 cells.

Fig. S2. MTS assay of TRIM44 knockdown NEC8 cells.

Fig. S3. Migration assay of TRIM44 knockdown NEC8 cells.

Fig. S4. Apoptosis assay in TRIM44 knockdown NEC8 cells.