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Trim47 overexpression correlates with poor prognosis in gastric cancer

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Trim47 is a member of the tripartite motif (TRIM) family that participates in many pathophysiological processes. However, the expression pattern and biological functions of *Trim47* in gastric cancer (GC) remain unclear. The present study aimed to further explore the clinicopathological significance and potential prognostic role of *Trim47* expression in GC. Therefore, in this study, *Trim47* mRNA level was investigated in the Cancer Genome Atlas (TCGA) and Oncomine database in GC. We detected *Trim47* mRNA and protein expression levels in GC and paired adjacent normal tissues. Kaplan-Meier method and Cox proportional hazard regression models were performed to analyze the survival of patients and prognostic factors. A gene set enrichment analysis (GSEA) was performed to determine the mechanism of *Trim47* in GC. Our results indicated that *Trim47* mRNA expression in GC tissues was significantly higher than adjacent normal tissues, as was *Trim47* protein expression. *Trim47* overexpression in GC tissues was significantly associated with tumor differentiation, T stage, N stage, M stage, and TNM stage. Kaplan-Meier analyses showed that high *Trim47* expression was associated with worse overall survival (OS) and disease-free survival (DFS) in GC patients. Multivariate analysis confirmed that *Trim47* expression was an independent prognostic factor for GC patients. Bioinformatics analysis and western blot indicated *Trim47* might regulate GC through NF-κB, EMT, hypoxia, and apoptosis signaling pathway in GC. Our results show that *Trim47* has the potential to serve as a novel prognostic biomarker in GC patients.

Key words: Trim47, gastric cancer, prognosis, immunohistochemistry, overall survival

Among cancers, gastric cancer (GC) has become a common malignant tumor in humans and a major health problem. GC ranks sixth for cancer incidence and third for cancer deaths with 834,000 deaths [1, 2]. Surgical resection is a curative treatment for early-diagnosed GC patients, but many patients are diagnosed at inoperable advanced stages or suffer from metastasis after resection [3–5]. The 5-year overall survival rate of GC patients is still very low due to the majority of patients already being at a progressive stage when diagnosed [6–8]. Therefore, there is an urgent need to identify novel biomarkers for early diagnosis, prediction of metastatic progression, and prognosis of GC patients.

Tripartite motif (TRIM) family proteins are members of the RING family of ubiquitin E3 ligases, which are characterized by three conserved domains, composed of RING, B-BOX, and coiled-coil (RBCC) [9–11]. TRIM family proteins participate in a wide array of cellular activities, such as cell cycle regulation, cell proliferation, migration, differentiation, apoptosis, inflammation, carcinogenesis, and immunity [12–19]. Trim47, a member of the TRIM family proteins, is localized to 17q24-25, a region that is frequently gained or amplified in multiple human tumor types [20]. Therefore, *Trim47* may also be associated with the progression and prognosis of human tumors. In addition, *Trim47* expression has been confirmed as a predictor of survival in patients with prostate cancer, non-small cell lung cancer, colorectal cancer, and breast cancer [21–24]. Nonetheless, it remains unclear whether *Trim47* regulates the progression and prognosis of GC.

Therefore, the present study was conducted to investigate the expression of *Trim47* in GC tissues and analyze its association with patient's clinical significance and prognosis.

Materials and methods

Clinical specimens. Clinical gastric cancer samples were obtained from the First Affiliated Hospital of Sun Yat-sen University between 2004 and 2008. 136 patients

with primary GC who had undergone gastric resection with lymph node dissection were randomly selected for this study. No patients received preoperative chemotherapy or radiation therapy. The diagnosis of GC was histologically confirmed by a pathologist. The stage of GC was determined according to the 8th edition of the tumor-node-metastasis (TNM) classification of malignant tumors established by the American Joint Committee on Cancer (AJCC) [25]. Another 30 GC tissues and paired adjacent normal tissues were obtained randomly by surgery from GC patients in 2019. The study was approved by the Ethical Review Committee of the First Affiliated Hospital of Sun Yat-sen University.

Cell culture, reagents, and transfection. The human GC cell line (AGS) was purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). The cell line was cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), appropriate amounts of penicillin (100 U/ml) and streptomycin (100 μg/ml), at 37 °C in air containing 5% CO_2. Antibodies against Trim47 (#26885-1-AP), HIF1a (#20960-1-AP), Vimentin (#60330-1-lg), E-Cadherin (#20874-1-AP), and GAPDH (#60004-1-lg) were purchased from Proteintech Group (Wuhan, China). Antibody against Bcl2 (#ab32124) was purchased from Abcam (Cambridge, MA, USA). Antibodies against N-Cadherin (#13116), P65 (#8242), P-P65 (#3033), and Caspase-3 (Cleaved-Caspase-3) (#9662) were purchased from Cell Signaling Technology (Danvers, MA, USA).

The *Trim47* siRNA and negative control siRNA (siNC) were purchased from RiboBio (Guangzhou, China). Cells were transfected with *Trim47* siRNA or negative control siRNA using Lipofectamine 3000 according to the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR). Total RNA was extracted using TRIzol reagent (Invitrogen) and reversely transcribed to cDNA following the manufacturer's instructions. Primers for qRT-PCR were used as follows: *Trim47* forward primer 5'-GCTTCAGGAGGCTGAGCAGT-3', reverse primer 5'-TCTGCTACGGCTGCACTCTT-3', GAPDH forward primer 5'-ATGTTGCAACCGGGAAGGAA-3', reverse primer 5'-AGGAAAAGCATCACCCGGAG-3'. The SYBR Premix Ex Taq™ II and CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, CA, USA) were used for qPCR assays.

Western blot analysis. Total protein was extracted from tissues or cells. Protein samples were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). After blocking with 5% skim milk in TBST, membranes were incubated with specific primary antibodies overnight at 4 °C. Subsequently, the membranes were incubated with HRP-conjugated secondary antibody. Protein bands were detected with the enhanced chemiluminescence reagent (Millipore, Billerica, MA, USA).

Immunohistochemistry (IHC) staining. The consecutive sections 4 μm in thickness were cut from each tissue-array block and used for IHC staining. The sections were deparaffinized, rehydrated, and boiled in a microwave oven with citrate buffer (pH 6.0) for 8 min for antigen retrieval. Endogenous peroxidase activity was blocked by incubating the slides in hydrogen peroxide (3%). The sections were blocked with normal goat serum to block nonspecific binding. Then sections were incubated with anti-*Trim47* antibody (1:100, Proteintech, 26885-1-AP) overnight at 4 °C. Subsequently, sections were incubated with an HRP-conjugated sheep anti-rabbit secondary antibody (DakoCytomation, Glostrup, Denmark) and diaminobenzidine (DAB). Finally, the sections were counterstained with hematoxylin, dehydrated, and mounted.

The result of the staining of *Trim47* for each section was assessed independently by two authors in a blinded manner according to the semiquantitative scoring system. First, no staining or staining of less than 10% of the tumor cells was considered negative (IHC score 0). Second, for more than 10% of the tumor cells, IHC was scored using the intensity of staining (1, weak staining; 2, moderate staining; 3, strong staining). *Trim47* expression was finally classified into low (0, 1) and high (2, 3).

Bioinformatics analysis. The RNAseq data of GC was downloaded from the Cancer Genome Atlas (TCGA) (https://genomecancer.ucsc.edu) and Oncomine databases (https://www.oncomine.org). GSEA v4.0.3 software was used to perform GSEA and analyze differences in the biologic pathway. *Trim47* mRNA expression level was separated into a high and a low group (critical value = median *Trim47* level). Functional gene sets were defined according to Molecular Signatures Database v7.1 (MSIGDB). A $p<0.05$ and FDR<0.25 were recognized as differentially expressed threshold.

Statistical analyses. Statistical analyses were performed using SPSS 22.0 (IBM Co, Chicago, IL, USA) and GraphPad Prism software 5.0 (GraphPad software, La Jolla, CA, USA). All data were presented as the mean ± SD. Student's t-test was used to determine the statistical differences between different groups. The relationships between *Trim47* expression and clinicopathological characteristics were compared by χ^2 test or Fisher exact test (two-sided). Survival curves were constructed using the Kaplan-Meier method, and differences between survival curves were determined to be significant based on the log-rank test. The Cox regression model analysis was performed to identify the independent prognostic factors of survival. A p-value <0.05 was considered statistically significant.

Results

Overexpression of *Trim47* in GC tissues. We analyzed TCGA databases through UALCAN [26] and found that the *Trim47* gene was upregulated in GC tissues compared with normal tissues (Figure 1A). The *Trim47* mRNA level in GC tissues (n=415) and normal tissues (n=34) from the TCGA cohort showed that *Trim47* mRNA expression was

significantly upregulated in GC tissues (p<0.001; Figure 1B). Compared with normal tissue, the *Trim47* mRNA level was also upregulated with different tumor stage (p<0.001; Figure 1C). Similar results were observed in other GC cohorts from the Oncomine database [27–30] (Figure 1D; Table 1). We detected *Trim47* mRNA expression level in 21 GC and paired adjacent normal tissues by qRT-PCR (Figure 1E) and protein expression level in GC and paired adjacent normal tissues by western blot (Figure 1F) and IHC staining (Figure 2). The results showed that *Trim47* mRNA and protein expres-

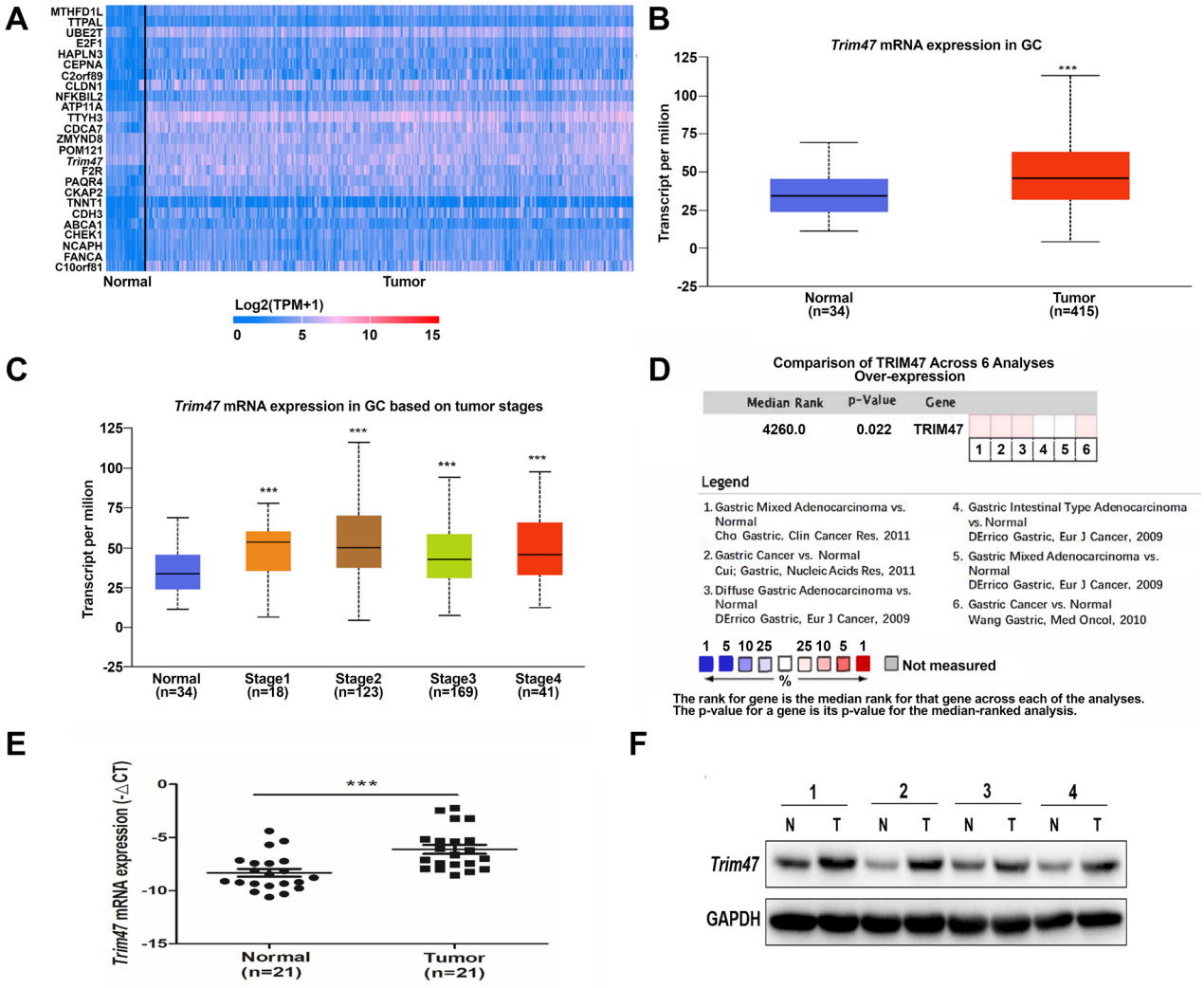

Figure 1. Increased *Trim47* expression in gastric cancer. A) Analysis of the TCGA data by a heat map. B) *Trim47* mRNA expression in GC and normal tissues from the TCGA database. C) *Trim47* mRNA expression based on tumor stage from the TCGA database. D) Six analyses were performed on the Oncomine database in comparing mRNA expression of *Trim47* between GC and normal tissues. E) Detection of *Trim47* mRNA expression level in GC and paired adjacent normal tissues by qRT-PCR. F) *Trim47* protein expression level was measured in GC and paired adjacent normal tissues by a western blot. The data are represented as mean ± SD. ***p<0.001

Table 1. Oncomine analysis of *Trim47* expression in gastric cancer (total of 4 GC cohorts).

Cohort	Sample (n)	t-test	Fold change	p-value
D'Errico et al. [30]	diffuse gastric adenocarcinoma (6) vs. normal (31)	2.387	1.585	0.013
	gastric mixed adenocarcinoma (4) vs. normal (31)	2.283	1.852	0.032
	gastric intestinal type adenocarcinoma (26) vs. normal (31)	2.232	1.668	0.015
Cui et al. [28]	gastric cancer (80) vs. normal (80)	1.747	1.216	0.041
Wang et al. [27]	gastric cancer (12) vs. normal (15)	2.221	1.459	0.018
Cho et al. [29]	gastric mixed adenocarcinoma (10) vs. normal (19)	2.043	1.372	0.026

sion levels were significantly higher in GC compared with adjacent normal tissues. Taken together, the above data showed that *Trim47* was upregulated in GC tissues.

Correlation between *Trim47* expression and clinicopathological characteristics in GC. To explore the association between *Trim47* expression and clinicopathological characteristics in GC patients, we performed IHC staining to detect *Trim47* expression in 136 GC specimens. As shown in Figure 2A, *Trim47* protein was mainly distributed in the cytoplasm. The relationships between *Trim47* expression and clinicopathological characteristics are summarized in Table 2. *Trim47* overexpression in GC tissues was significantly related to tumor differentiation (p=0.021), T stage (p=0.010), N stage (p=0.003), M stage (p=0.016), and TNM stage (p=0.001) (Table 2). Nevertheless, other clinical characteristics such as gender, age, tumor size, and CEA level were not associated with the expression of *Trim47*.

***Trim47* overexpression indicated a worse prognosis in GC patients.** The follow-up period of the 136 GC patients ranged from 1.7 to 152.5 months. The 5-year OS rate was 79.4% in the low *Trim47* expression group and 31.6% in the high *Trim47* expression group. Our data showed that high *Trim47* expression was associated with worse OS (p<0.001; Figure 3A) and DFS (p<0.001; Figure 3B) in GC patients. Furthermore, we also detected the prognostic value of *Trim47* expression in early (TNM stage I and II) and advanced (TNM stage III and IV) GC. The results indicated that high *Trim47* expression was associated with worse OS (p<0.05, Figures 3C, 3E) and DFS (p<0.05, Figures 3D, 3F) in both early and advanced GC.

To determine whether *Trim47* expression was an independent prognostic predictor for patients with GC, we performed univariate Cox regression analysis to assess the impact of *Trim47* expression level and other clinicopatho-

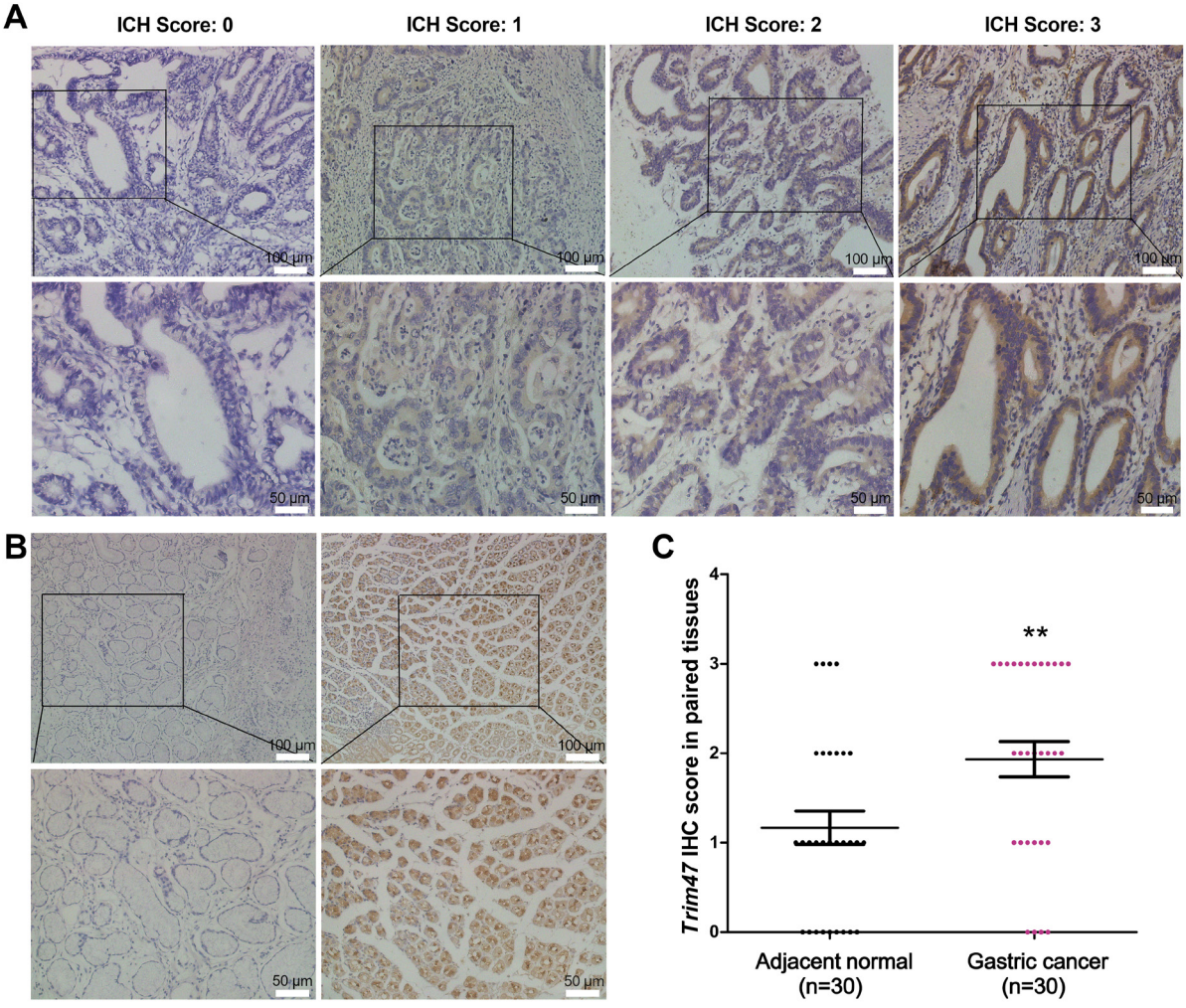

Figure 2. Detection of *Trim47* protein expression in GC tissues and adjacent normal tissues by IHC staining. A) IHC staining of *Trim47* protein in GC tissues. IHC scoring was performed according to the staining intensity (0, negative; 1, weak; 2, moderated; 3, strong). B) IHC staining of *Trim47* protein in adjacent normal tissues (left panel, negative; right panel, positive). C) Protein expression of *Trim47* was significantly higher in GC tissues compared with adjacent normal tissues by IHC (original views ×100, enlarged views ×200). The data are presented as mean ± SD.p<0.01**

Table 2. Relationship between *Trim47* expression levels and clinicopathological characteristics in GC patients.

Characteristics	N (136)	*Trim47* expression		χ^2	p-value
		Low (44)	High (92)		
Gender					
Male	92	32	60	0767	0.381
Female	44	12	32		
Age (years)					
≤60	75	24	51	0.010	0.922
>60	61	20	41		
Tumor size (cm)					
≤4	88	30	58	0.344	0.557
>4	48	14	34		
Tumor differentiation					
Well or moderate	58	25	33	5.340	**0.021**
Poor	78	19	59		
T stage					
T1/T2	58	25	31	6.570	**0.010**
T3/T4	80	19	61		
N stage					
N0	42	21	21	8.647	**0.003**
N1/N2/N3	94	23	71		
M stage					
M0	111	41	70	5.798	**0.016**
M1	25	3	22		
TNM stage					
I/II	50	25	25	11.251	**0.001**
III/IV	86	19	67		
CEA level (μg/l)					
<5	110	38	72	1.264	0.261
≥5	26	6	20		

Table 3. Univariate and multivariate Cox regression analysis for OS.

Characteristics	Univariate analysis		Multivariate analysis	
	HR (95%CI)	p-value	HR (95%CI)	p-value
Gender	0.683 (0.430–1.084)	0.106		
Age	1.123 (0.717–1.758)	0.613		
Tumor size	1.376 (0.874–2.166)	0.169		
Tumor differentiation	1.575 (0.990–2.505)	0.055		
T stage	2.440 (1.471–4.048)	**0.001**		
N stage	2.184 (1.291–3.695)	**0.004**		
M stage	6.678 (3.838–11.620)	**<0.001**	3.407 (1.886–6.154)	**<0.001**
TNM stage	3.172 (1.878–5.356)	**<0.001**	1.823 (1.019–3.261)	**0.043**
CEA concentration	1.419 (0.825–2.440)	0.207		
Trim47 expression	4.186 (2.313–7.575)	**<0.001**	2.927 (1.559–5.494)	**0.001**

logical factors on the prognosis of GC patients. Univariate analysis revealed that clinical variables including T stage, N stage, M stage, TNM stage, and *Trim47* expression were significantly associated with OS (Table 3). Furthermore, multivariate Cox analysis showed that *Trim47* expression was an independent predictor of OS in GC patients (HR=2.927, 95% CI: 1.559–5.494, p=0.001, Table 3). Moreover, *Trim47* expression was also an independent predictor of DFS in GC patients (HR=3.382, 95% CI: 1.795–6.373, p<0.001, Table 4). These results indicated that high *Trim47* expression was an independent prognostic predictor for GC patients.

The main enriched pathways in GC tissues with high *Trim47* expression. GSEA is a microarray data analysis method that uses predefined gene sets and ranks of genes to identify more subtle changes of gene expression [31, 32]. In this study, to explore the role of *Trim47* in GC prolifera-

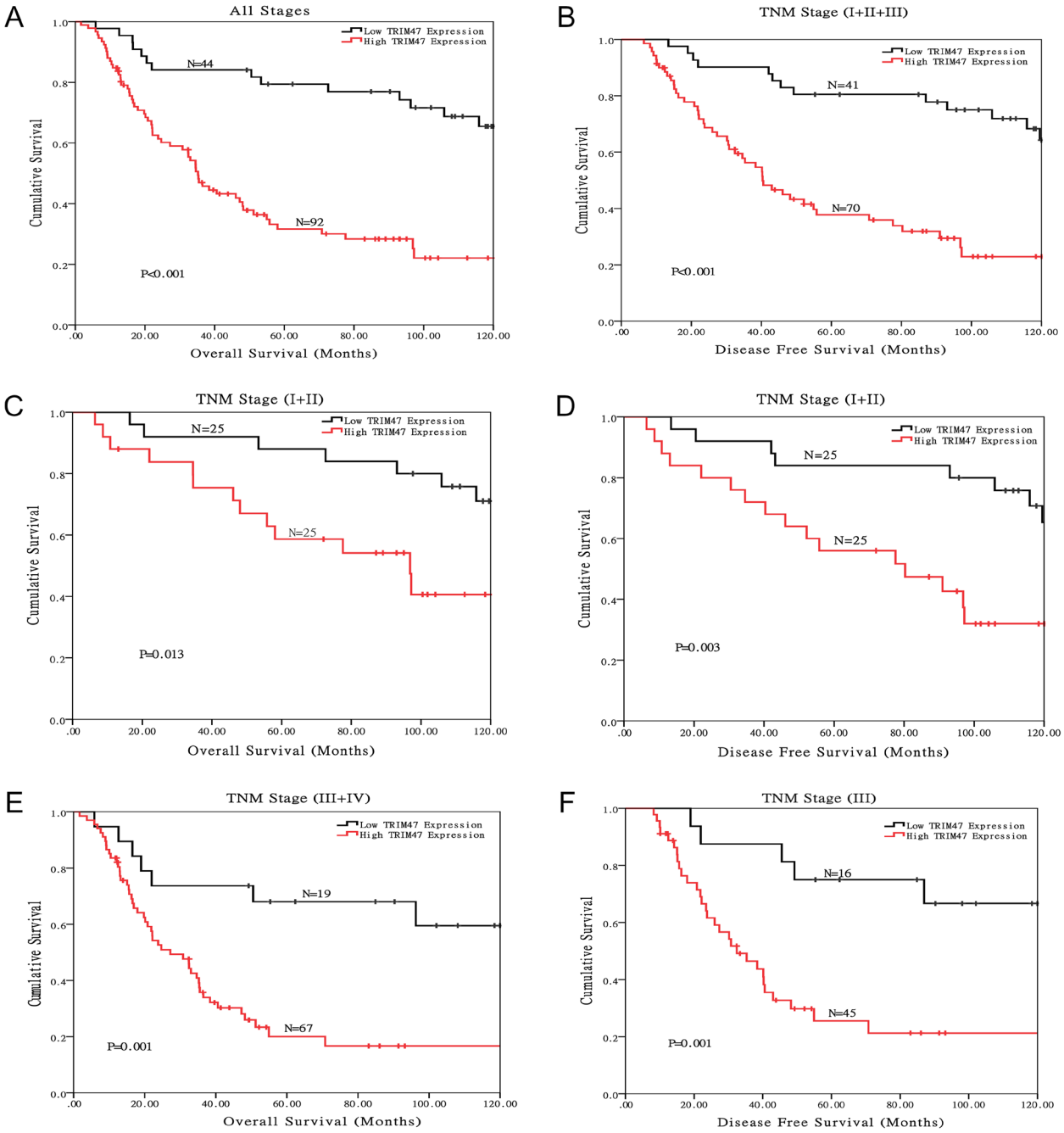

Figure 3. High *Trim47* expression in tumors predicted poor prognosis in GC patient. GC patients with high *Trim47* expression had worse OS A) and DFS B) than those with low *Trim47* expression. Patients with high *Trim47* expression had worse OS C) and DFS D) in early GC. Patients with high *Trim47* expression had a worse OS (E) and DFS (F) in advanced GC.

tion and metastasis, an integrative analysis of GC microarray was carried out on the base of the TCGA database. The GSEA results showed that the high *Trim47* expression group was significantly enriched in NF-κB, epithelial-to-mesenchymal transition (EMT), hypoxia, and apoptosis signaling pathway (Figures 4A–4D). We further confirmed that *Trim47* regulated GC through NF-κB, EMT, hypoxia, and apoptosis signaling pathway by western blot. We established *Trim47* knockdown in AGS cells to ascertain the roles of *Trim47* in signaling pathway (Figure 4E). As to EMT, we performed a western blot to measure the expression of EMT markers in our cell model. Then we found that the epithelial marker E-cadherin was elevated significantly in *Trim47* knockdown cells, while the mesenchymal markers

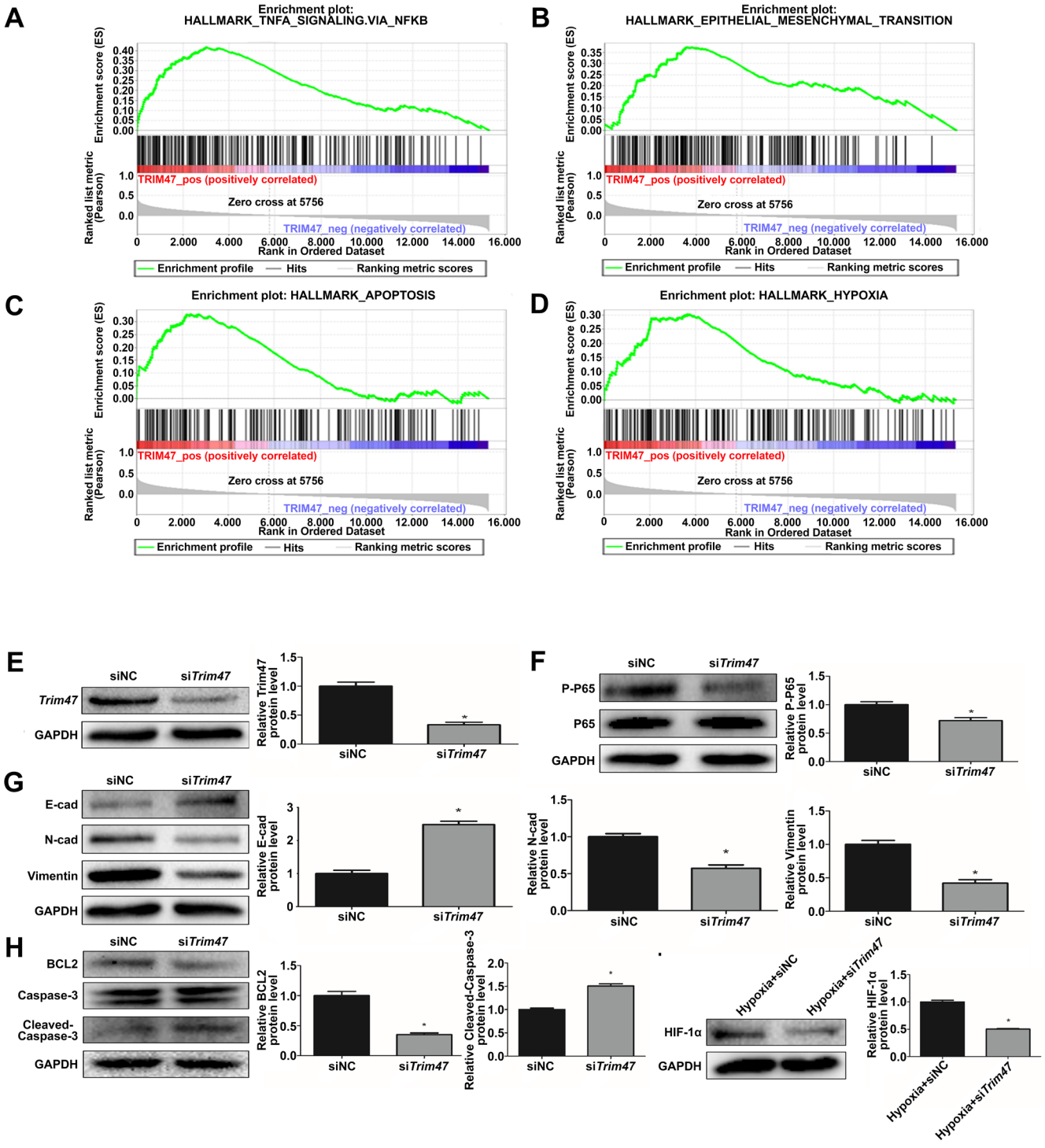

Figure 4. The main enriched pathways. A) High expression of *Trim47* was enriched in the NF-κB pathway by GSEA analysis. B) High expression of *Trim47* was enriched in the EMT pathway by GSEA analysis. C) High expression of *Trim47* was enriched in the apoptosis pathway by GSEA analysis. D) High expression of *Trim47* was enriched in the hypoxia pathway by GSEA analysis. E) The effect of *Trim47* knockdown level was confirmed by western blot analysis in AGS cells. F) Western blot analysis of P-P65 and P65 in *Trim47* knockdown and control AGS cells. G) Western blot analysis of E-cadherin, N-cadherin, and Vimentin in *Trim47* knockdown and control AGS cells. H) Western blot analysis of Bcl2, Caspase-3, and cleaved Caspase-3 in *Trim47* knockdown and control AGS cells. I) Western blot analysis of HIF1a in *Trim47* knockdown and control AGS cells in hypoxic conditions. The data are presented as mean ± SD. *p<0.05

Table 4. Univariate and multivariate Cox regression analysis for DFS.

Characteristics	Univariate analysis		Multivariate analysis	
	HR (95%CI)	p-value	HR (95%CI)	p-value
Gender	0.684 (0.409–1.144)	0.148		
Age	1.244 (0.757–2.046)	0.389		
Tumor size	1.641 (0.996–2.704)	0.052		
Tumor differentiation	1.795 (1.071–3.006)	**0.026**	1.841 (1.088–3.115)	**0.023**
T stage	2.278 (1.336–3.885)	**0.003**	1.958 (1.123–3.416)	0.018
N stage	1.769 (1.031–3.034)	**0.038**		
TNM stage	1.994 (1.193–3.333)	**0.008**		
CEA concentration	1.513 (0.818–2.797)	0.187		
Trim47 expression	4.086 (2.222–7.513)	**<0.001**	3.382 (1.795–6.373)	**<0.001**

N-cadherin and Vimentin were downregulated. The result showed that *Trim47* knockdown inhibited the GC cell EMT process. We further confirmed the effect of *Trim47* on NF-κB, hypoxia, and apoptosis signaling pathway in GC. The results suggested that *Trim47* knockdown decreased P-P65, Bcl2, and HIF1a levels and promoted the cleaved Caspase-3 level. The findings indicated that the expression of *Trim47* was significantly associated with NF-κB, EMT, hypoxia, and apoptosis signaling pathway.

Discussion

TRAM proteins often form a medium-sized subfamily of mainly post-translational modification modifiers, including ubiquitylation and other ubiquitin-like modifications [33, 34]. Therefore, TRIM proteins are involved in inflammatory and innate immune responses, especially in carcinogenesis and progression of various tumors [13, 35]. Increasing evidence had demonstrated that *Trim47* was upregulated in tumor tissue and might serve as a new prognostic marker in patients with malignant tumors [21–24]. Fujimura et al. found that a higher *Trim47* expression in prostate cancer tissues was significantly correlated with advanced pathologic T stage and worse cancer-specific survival rates [21]. Similarly, elevated *Trim47* expression was also associated with tumor progression in breast cancer, non-small cell lung carcinoma, and colorectal cancer [22–24]. However, there was no study to explore the relationship between *Trim47* expression and prognosis of GC.

In the present study, we identified that *Trim47* could be a potential new biomarker to diagnose and evaluate the prognosis of GC patients. First, we analyzed the data from TCGA and Oncomine database to prove that the level of *Trim47* mRNA in GC tissues was significantly higher than that in normal tissues. We further confirmed the above result by qRT-PCR in GC tissues and paired adjacent normal tissues. Second, we further assessed *Trim47* protein expression by western blot and IHC staining in GC tissues and paired adjacent normal tissues. The protein expression of *Trim47* was significantly higher in GC compared with adjacent normal tissues. Third, we performed IHC staining and analyzed the relationship between *Trim47* expression and prognosis in 136 GC patients. The result showed that higher *Trim47* expression was associated with worse OS and DFS in GC patients. Finally, the multivariate Cox regression analysis demonstrated that *Trim47* expression was an independent predictor of OS and DFS in GC patients.

The findings of this study also revealed that *Trim47* overexpression was significantly related to tumor differentiation, T stage, N stage, M stage, and TNM stage. The results of GESA showed that the expression of *Trim47* influenced NF-κB, EMT, hypoxia, and apoptosis signaling pathway in GC. Previous studies also had demonstrated that upregulated *Trim47* stimulated cell proliferation and metastasis via exerting an inhibitory effect on P53 and a facilitatory effect on the NF-κB signaling pathway in non-small cell lung carcinoma [22]. Knockdown of *Trim47* in breast cancer cells inhibited cell proliferation, migration, and invasion by inhibiting the activation of the PI3k/AKT signaling pathway [24]. Besides, knockdown of *Trim47* also inhibited epithelial-to-mesenchymal transition (EMT) in breast cancer [24]. *Trim47* promoted colorectal cancer development by upregulating the expression of CCL15 and CCR1 via interacting with SMAD4 and enhancing ubiquitylation and degradation of SMAD4 [23]. We further confirmed *Trim47* regulates GC through NF-κB, EMT, hypoxia, and apoptosis signaling pathway by western blot. The result indicated that the expression of *Trim47* was significantly associated with NF-κB, EMT, hypoxia, and apoptosis signaling pathway.

In conclusion, our study revealed that elevated expression level of *Trim47* was significantly associated with poor OS and DFS in GC patients. Moreover, the expression level of *Trim47* was associated with clinicopathological features including tumor differentiation, T stage, N stage, M stage, and TNM stage. Our findings suggest that *Trim47* could be a potential new biomarker to evaluate the prognosis of GC patients.

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