



Overexpression of TWA1 predicts poor prognosis in patients with gastric cancer



Jianbo Xiong^a, Zongfeng Feng^a, Zhengrong Li^{a,*}, Tao Zhong^a, Zhouwen Yang^a, Yi Tu^b, Tao Xiao^a, Zhigang Jie^a, Yi Cao^{a,*}

^a Department of Gastrointestinal Surgery, the First Affiliated Hospital of Nanchang University, Nanchang 330006, Jiangxi Province, China

^b Department of Pathology, the First Affiliated Hospital of Nanchang University, Nanchang 330006, Jiangxi Province, China

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ABSTRACT

TWA1 is associated with microtubule dynamics, cell migration, nucleokinesis and chromosome segregation. However, the role of TWA1 in gastric cancer (GC) remains unclear. In this study, Cosmic database revealed that the expression level of TWA1 ranks in the top 20 of overexpressed genes in GC. Further bioinformatic analysis revealed that the expression level of TWA1 was not in connection with the infection status of HP or EB. IHC and IF showed that TWA1 protein was present in both the cytoplasm and nucleus, but mainly in the cytoplasm. The high expression level of TWA1 was also related to tumor size, depth of invasion, lymph node metastasis, TNM stage, cancerous node and vascular invasion. Furthermore, higher TWA1 expression was also associated with shorter PFS and OS in GC. The univariate and multivariate analysis suggested the expression of TWA1 was an independent poor prognostic factor in GC. DNA copy number gain contributes to TWA1 overexpression and promoter methylation of TWA1 predicts profitable prognosis. Co-expression showed that TAF4 may function as a transcription factor (TF) regulates TWA1 expression, which further to mediate tumor invasion and metastasis. These findings revealed that TWA1 plays an important role in the development of GC and is expected to become an important biomarker and therapeutic target of tumors.

1. Introduction

Gastric cancer (GC) is the fourth most common malignant tumors and the second leading cause of cancer-associated mortality worldwide [1,2]. Despite substantial advances in surgery, chemotherapy, radiotherapy and targeted molecular therapy, the clinical consequence of GC patients suffering from tumor invasion and metastasis remains to be improved, and the 5 years survival rate is only 10%–30% [3,4]. Therefore, identifying novel diagnostic and prognostic markers, and exploring the underlying molecular mechanism of tumorigenesis and metastasis is critical for preventing and treating GC.

TWA1 (Two hybrid associated protein No.1 with RanBPM), also known as GID8 (glucose-induced degradation protein 8 homolog) and C20orf11, which was isolated as a protein can interact with Ran binding protein by M. Umeda in 2002 [5]. TWA1 is well conserved through evolution and is consist of LisH, CTLH and CRA domains. Due to its CTLH complex, TWA1 is often described as an E3 ubiquitin ligase complex involved in protein degradation through ubiquitination but also via vacuole-dependent degradation [6]. As one of the components

of the mammalian muskelin/RanBP9/CTLH complex, TWA1 is detected in proteins involved in microtubule dynamics, cell migration, nucleokinesis and chromosome segregation [7,8]. However, the function of TWA1 has not yet to be fully evaluated. Researches had reported TWA1 may act to down-regulate gluconeogenesis in budding yeast and share LisH and CTLH domain with RanBPM, MAEA and Rmnd5 by phylogenetic and sequence analyses [8]. It also had been reported that TWA1 was significantly upregulated in human colorectal cancer (CRC) tissues, correlating with the nuclear accumulation of β -catenin and poor survival of CRC patients [9]. Depletion of TWA1 attenuates Wnt-stimulated gene expression, dorsal development of zebrafish embryos and xenograft tumor growth of CRC cells [9]. These findings indicate that TWA1 may function as an oncogene and contribute to many physiological and pathological processes. However, the exact role of TWA1 in GC and the potential molecular mechanism have not yet been fully elucidated.

In the present study, we found that TWA1 was upregulated in GC tissues by bioinformatics analysis, which was verified in paired GC tissues by qRT-PCR, Western Blot (WB) and GC tissues by

* Corresponding authors at: Department of Gastrointestinal Surgery, the First Affiliated Hospital of Nanchang University, No. 17 Yongwai Zheng Road, Nanchang 330006, Jiangxi Province, China.

E-mail addresses: lzr13@foxmail.com (Z. Li), doctorcaoyi@126.com (Y. Cao).

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immunohistochemical (IHC). TWA1 was also associated with poor progression-free survival (PFS) and overall survival (OS) in GC. In addition, the present experiment also investigates the biological function and mechanism of TWA1 in patients with GC. All data presented herein suggest that TWA1 maybe a promising molecular target for the therapy of GC and may play a vital role in the progression of GC.

2. Materials and methods

2.1. Bioinformatic analysis

Cosmic (Catalogue of Somatic Mutations in Cancer) database (<https://cancer.sanger.ac.uk/cosmic/>) was used to analysis the up-regulation gene and the mutation of TWA1 in GC. The Oncomine database (<https://www.oncomine.org/resource/login.html>) was searched to determine the transcription level of TWA1 gene in GC. The Ualcan database (<http://ualcan.path.uab.edu>) was used to analysis TWA1 mRNA expression from the TCGA database. cBioportal (<https://www.cbioportal.org>) was applied to investigate the gene positively associated with TWA1 expression in GC. GEPIA database GEPIA database (<http://gepia.cancer-pku.cn>) was used to explore the expression of gene and the co-expression of gene. ChIPBase (<http://rna.sysu.edu.cn/chipbase/>) is an open database for studying the transcription factor binding sites and motifs. TIMER database (<https://cistrome.shinyapps.io/timer/>) was used to estimate the differential expression between tumor and adjacent normal tissues of TWA1 across all TCGA tumors.

2.2. Patients and tissue samples

The present study was approved by the Ethics and Committee of the First Affiliated Hospital of Nanchang University (Nanchang, China) (No. 087). In total, 201 paraffin-embedded human GC tissues and 60 adjacent normal tissues between June 2011 and December 2012 were collected from the Pathology Department of the First Affiliated Hospital of Nanchang University. The median age of patients (62 women and 139 men) was 57.4 years (range from 20 to 80). A total of 32 paired GC tissues and noncancerous gastric mucosal tissues were collected from GC patients who underwent surgery in the First Affiliated Hospital of Nanchang University (Nanchang, China) from January 2017 to December 2018. Liquid nitrogen was used to immediately snap-frozen all the fresh samples, which were store at -80°C freezer for long time. All samples were obtained with informed consent and none of the patients had a history of radiotherapy or chemotherapy prior to surgery. The tumor-node-metastasis staging system was performed according to the 2018 American Joint Committee on Cancer criteria (8th AJCC TNM GC stage) and the histological differentiation was based on World Health Organization criteria.

2.3. Immunohistochemistry (IHC)

The formalin-fixed paraffin-embedded tumor and normal tissues sections (4- μ m) were deparaffinized in xylene and rehydrated in different concentrations of alcohol and distilled water, followed by microwave antigen retrieval. The sections were then incubated with rabbit anti-human polyclonal TWA1 antibody (catalog no. ab106453; Abcam, Cambridge, UK) in a humidified chamber overnight at 4°C. Then slides were washed 3 times in PBS for 5 min each time. The anti-rabbit antibodies were incubated at room temperature for 50 min and followed washing in PBS 3 times. An appropriate amount of diaminobenzidine (DAB) color solution was added to the circle to cover the tissue completely. The results were observed under an optical microscope by two independent pathologists. TWA1 expression levels were scored according to the proportion of stained tumor cells and the intensity of the staining [10]. The staining intensity was graded as follows: 0 (negative, -), 1 (weak, +), 2 (moderate, ++), and 3 (strong, +++). The proportion of staining was graded as follows: 0 (0–5%); 1 (5–25%); 2

(25–50%); 3 (50–75%) and 4 (75–100%). The final TWA1 expression scores were calculated by multiplying the above two scores. Slides were considered low or high, with scores of <6 or \geq 6, respectively.

2.4. RNA extraction and reverse transcription-quantitative polymerase chain reaction (qRT-PCR)

Total RNA was extracted from GC tissues and paired noncancerous gastric mucosal tissues using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, M, USA). cDNA was obtained from the RNA using an EasyScript First-Strand cDNA Synthesis Supermix kit (TransGen Biotech, Inc., Beijing, China) and qRT-PCR analysis was performed on an ABI Prism 7500 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR Green qPCR Supermix-UDG with ROX (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The internal control was human β -actin mRNA.

The primer was: β -actin, forward 5'-CATCCGCAAAGACCTGTACG-3'; reverse 5'-CTGCTTGCTGATCCACATC-3'; TWA1, forward 5'-GCAGAAAAACCCGATGAAATCAC-3'; reverse 5'-AGATCCACACTAGGTTTCGATTCC-3'. TWA1 expression was normalized to β -actin and relative expression levels were calculated.

2.5. Western blot analysis

Total proteins were extracted from fresh tissues using RIPA lysis buffer (Applygen, Beijing, China) supplemented with 1% protease inhibitors and 1% phosphatase inhibitors (CW BIO Beijing, China). Protein lysates were separated on 10% sodium dodecyl sulfate polyacrylamide gels and transferred to nitrocellulose filter membrane using the semi-dry technique. The membranes were blocked in 5% nonfat milk supplement with TBST (tris-buffered saline with Tween-20) for 1 h at room temperature. After incubating overnight at 4°C with primary antibodies against TWA1 (1:1000 dilution, Abcam, catalog no.ab106453, Abcam) and β -actin (1:1000 dilution, ZSGB-BIO, China), the membranes were washed 3 times in TBST for 10 min. Then the membranes were incubated with the corresponding horseradish peroxidase conjugated secondary antibodies at room temperature for 1 h. After washing 3 times for 10 min with TBST, the target proteins were stained using a chemiluminescence detection system (Thermo Fisher Scientific, Inc.).

2.6. Immunofluorescence (IF) analysis

Immunofluorescence analysis was performed according to previously study [37,38]. Primary antibody against TWA1 (1:50, Abcam, Cambridge, UK). Secondary antibody (Alexa Fluor 647, Abcam, Cambridge, UK). 4', 6-diamidino-2-phenylindole (DAPI, sigma) was used for cell nuclei stained. Confocal laser-scanning microscope (Zeiss LSM700) was performed for location detection.

2.7. Statistical analysis

Data were analyzed using paired or unpaired t-tests to compare quantitative variables. The comparison of TWA1 between the carcinoma tumor and normal tissues were compared using the Mann-Whitney U test. Chi-squared tests or Fisher's exact test were adopted to analyze the association between amount of TWA1 and clinicopathological characteristics. A survival curve was plotted by the Kaplan-Meier method and a log-rank test was used to compare the survive curves. Univariate and multivariate analysis were plotted by a Cox proportional hazards model. All statistical analyses were performed using SPSS 23.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 7 software (Graphpad, Inc., La Jolla, CA, USA). $P < 0.05$ was considered as statistically significant.

Fig. 1. Overexpression of TWA1 in GC by bioinformatics analysis. A. The top 20 ranked genes (rows) with mRNA over expression in gastric cancer (each column represents a TCGA sample) from Cosmic database. TWA1 was also known as C20orf11. More details can be found in the website. B. Relative expression of TWA1 mRNA in human GC tissues (Tumor, n = 408) and gastric normal tissues (Normal, n = 211) from GEPIA database. C. Relative expression of TWA1 in normal gastric tissues and different pathological types of GC. Normal (n = 29); Diffuse-: diffuse gastric adenocarcinoma (n = 13); adeno-: gastric adenocarcinoma (n = 14); Intestinal-: gastric intestinal type adenocarcinoma (n = 61); Mixed: gastric mixed adenocarcinoma (n = 8). D. Relative expression of TWA1 in GC (Tumor, n = 96) and normal gastric tissues (Normal, n = 29) ($P < 0.05$). E. Relative expression of TWA1 between the HP (-) (n = 39) and HP (+) (n = 43); EB (-) (n = 74) and EB (+) (n = 10) ($P > 0.05$). (The data of C, D and E was from Oncomine database, Chen gastric, IMAGE 132066). F. Relative expression of TWA1 in normal tissues and different TNM stages of GC from Ualcan database. G. TWA1 expression elevated in cholangio carcinoma (CHOL), colon adenocarcinoma (COAD), liver hepatocellular carcinoma (LIHC), rectum adenocarcinoma (READ), lung adenocarcinoma (LUAD) and so on from TIMER database. The gray columns represent a statistically significant difference between the tumors and the normal tissues of gene expression. P-value Significant Codes: $0 \leq *** < 0.001 \leq ** < 0.01 \leq * < 0.05$.

3. Results

3.1. Overexpression of TWA1 in GC by bioinformatics analysis

Cosmic [11] was used to analyze the differential expression genes (DEGs) in GC and the results showed that the majority of top 20 genes with somatic mutations which are mRNA over expression and CNA gain, including TWA1 (Fig. 1A). To further investigate the role of TWA1 in GC, GEPIA database [12] was used to analyze the expression. A total of 408 primary GC tissues and 211 normal gastric tissues were analyzed and TWA1 mRNA was upregulated in GC tissues than normal tissues (Fig. 1B). TWA1 was determined to be elevated in multiple GC types, compared with the normal tissue, including adenocarcinoma, intestinal carcinoma, mixed carcinoma from Oncomine Chen gastric database [13] (Fig. 1C). Further statistical analysis showed that the expression of TWA1 in cancer tissues was significantly higher than that in normal tissues in Oncomine Chen gastric database which include 96 GC tumor tissues and 29 gastric normal tissues (Fig. 1D). However, we did not find significant difference between the positive and negative status of helicobacter pylori (HP) and Epstein-Barr virus (EB) for the expression of TWA1 (Fig. 1E). TWA1 mRNA expression in different stages of GC showed no significant difference, but TWA1 expression in either stage was higher than that in normal gastric tissues from the Ualcan database [14] (Fig. 1F). To further investigate the TWA1 expression profile in difference human cancer, we searched in TIMER database [15] and found that TWA1 elevated in cholangio carcinoma (CHOL), colon adenocarcinoma (COAD), liver hepatocellular carcinoma (LIHC), rectum adenocarcinoma (READ), lung adenocarcinoma (LUAD) and so on (Fig. 1G).

3.2. Aberrant expression of TWA1 in GC tissues

To characterize the expression of TWA1 in the progression of GC, immunohistochemistry was performed on 201 primary GC tumor tissues and 60 adjacent non-tumor tissues. TWA1 protein was found in both the cytoplasm and nucleus, but mainly in the cytoplasm, and the staining in non-cancer tissues was weaker or negative (Fig. 2A). Tissue immunofluorescence also showed that the TWA1 protein was mainly localized on the cytoplasm, which also revealed TWA1 overexpressed in GC tissues than the adjacent normal tissues (Fig. 2E). Then we scored the results of IHC among these specimens (Fig. 2B). A significant up-regulation in the expression of TWA1 was observed in 58.2% (117/201) of the GC tissues, compared with the normal tissues (15.0%, 9/60), and this difference was statistically significant ($P < 0.05$).

The present study investigated the association between the expression of TWA1 and the clinicopathological features of GC (Table 1). A high expression level of TWA1 was correlated with tumor size ($P < 0.01$), depth of invasion ($P < 0.01$), tumor-node-metastasis (TNM) stage ($P < 0.01$), lymph node metastasis ($P < 0.01$), cancerous node ($P < 0.01$) and vascular invasion ($P < 0.01$). However, no correlation was found between the expression of TWA1 and other variables, including age ($P = 0.671$), gender ($P = 0.065$), tumor location ($P = 0.275$), tumor differentiation ($P = 0.144$) and nerve invasion ($P = 0.885$).

The mRNA and protein expression of TWA1 were detected in randomly selected 32 pairs of GC tissues and adjacent non-tumor tissues by

qRT-PCR and Western blot. As shown in Fig. 2F, TWA1 protein expression in the tumor tissues was higher than the adjacent nontumor tissues. Similarly, the results of TWA1 mRNA expression also showed the same phenomenon ($P < 0.05$) (Fig. 2G). These results indicated that TWA1 in the GC tissues was up-regulated at the level of transcription.

3.3. Association between TWA1 and prognosis

Considering TWA1 was high expressed in GC tissues than that of matched normal tissues and closely associated with TNM stage and lymphatic metastasis, it was reasonable to investigate an association between TWA1 protein levels and overall survival (OS). Kaplan-Meier survival analysis and log-rank test were performed for all the 201 patients. The results revealed that increased TWA1 expression correlated with poor PFS and OS ($P < 0.05$, Fig. 2C and D). The overall median survival was 65 months in low or no TWA1 expression group compared to 27 months in high TWA1 expression group ($P < 0.01$).

Both univariate and multivariate analysis were used to investigate the relationship between TWA1 expression and prognostic factors in GC (Table 2). Univariate analysis indicated that high TWA1 expression was significantly correlated with poor OS ($P < 0.01$). This correlation was also observed in other prognostic marker including tumor size ($P < 0.01$), tumor location ($P < 0.01$), depth of invasion ($P < 0.01$), lymph node metastasis ($P < 0.01$), TNM stage ($P < 0.01$), cancerous node ($P < 0.01$) and vascular invasion ($P < 0.01$). Furthermore, multivariate analysis revealed that TWA1 expression in GC was an independent poor prognostic marker for OS. Additionally, depth of invasion, TNM stage and cancerous node were also independent prognostic factors for OS.

3.4. DNA copy number gain contributes to TWA1 overexpression and promoter hypermethylation of TWA1 predicts favorable prognosis

TWA1 resides on the long arm of chromosome 20 (20q13.33) and contains 5 exon count, which encoded protein 228 amino acids and weight 27 kDa (Fig. 3A). As shown in Fig. 3B, compared to ERBB2, PTEN, CCND1, MYC and APC, TWA1 presented overexpression in GC with amplification and mRNA upregulation (Fig. 3B). Considerate that CNA gain and DNA methylation are two main reasons for gene mRNA overexpression, the TCGA gastric data was download from cBioportal database and analyzed. There was no significant association between TWA1 mRNA expression and DNA methylation ($P = 0.43$) (Fig. 3C). However, TWA1 mRNA expression was positively correlated with DNA copy number gain in GC tissues ($P < 0.01$) (Fig. 3D). These results revealed that DNA copy number gain, but not the DNA methylation may be the main reason for TWA1 overexpression.

From the above analysis, we knew that DNA methylation may not affect TWA1 expression. Amazing, we found there are many CpG islands in the promoter of TWA1 by MethPrimer [16] (Fig. 3E), which means DNA methylation more likely to occur. Promoter DNA methylation, which occurs on cytosine nucleotides across CpG islands, results in gene silencing and represents a major epigenetic alteration in human cancer [17]. Abnormal hypomethylation of oncogenes is one of the characteristics of tumor cells [18]. However, as shown in Fig. 3F, the methylation level of TWA1 is not differ significantly in GC with different stages. Kaplan-Meier survival analysis by MethSuv [19] showed

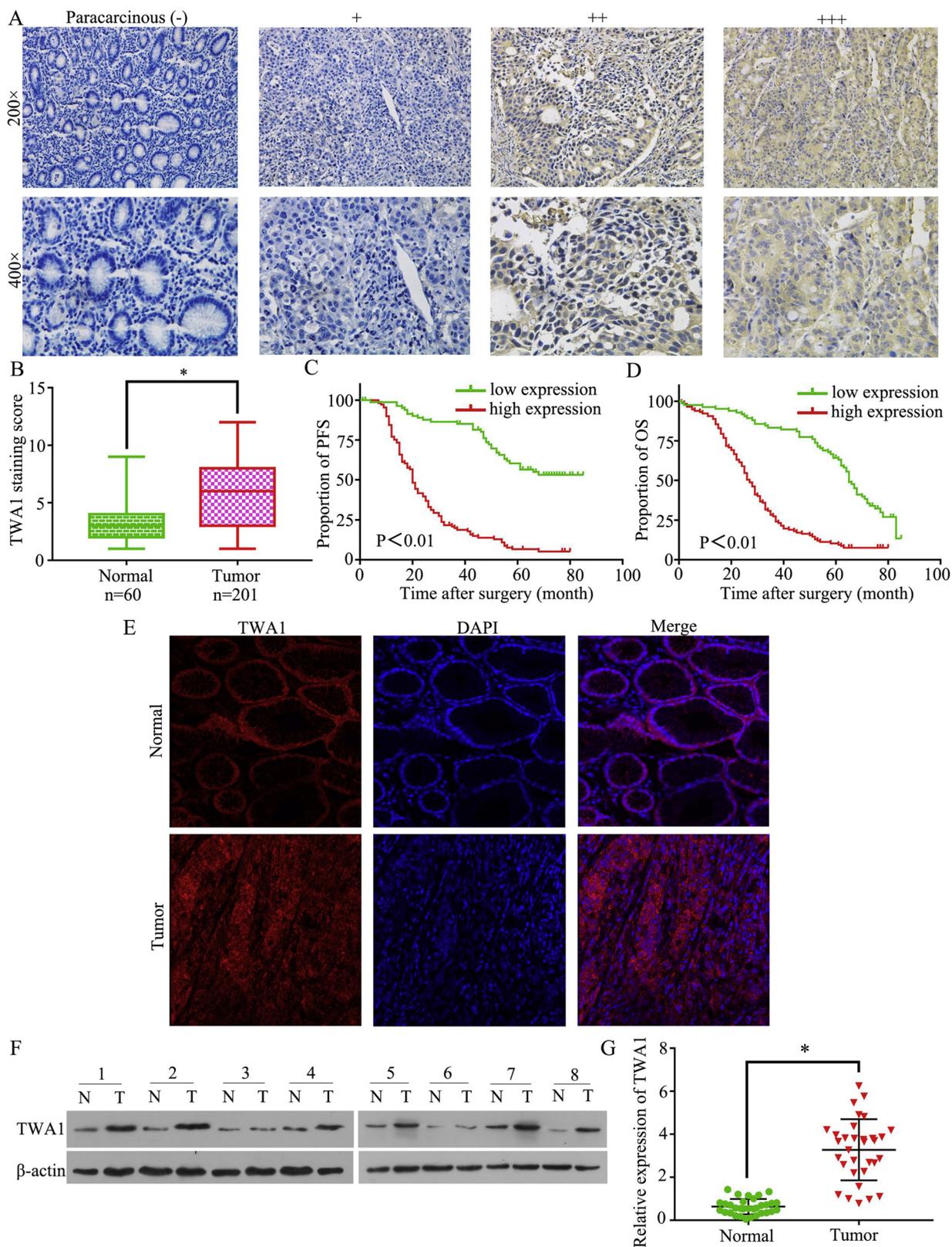


Fig. 2. TWA1 was overexpressed in GC tissues and associated with prognosis. A. Immunohistochemistry (IHC) revealed TWA1 expression in GC tissues (n = 201) and adjacent non-tumor tissues (n = 60). B. TWA1 staining score in GC tissues (Tumor) and non-tumor tissues (Normal) by IHC analysis. C. Progression free analysis (PFS) of GC patients with high or low expression of TWA1 (n = 201). D. Overall Survival analysis (OS) of GC patients with high or low expression of TWA1 (n = 201). E. Tissue immunofluorescence (IF) analyzed the localization of TWA1 protein in paired GC tissues. F. The expression of TWA1 in paired GC tissues analyzed by Western Blot (n = 32). G. qRT-PCR analyzed the expression of TWA1 in paired GC tissues (n = 32) ($P < 0.05$).

Table 1
Association between the expression of TWA1 and clinicopathological parameters of patients with GC.

Variable	n	Expression of TWA1		P-value
		Low (<6)	High (≥6)	
Age (years)	201			
< 60	104	45	59	0.671
≥ 60	97	39	58	
Gender				
Male	139	52	87	0.065
Female	62	32	30	
Tumor size (cm)				
< 4	110	63	47	< 0.01
≥ 4	91	21	70	
Tumor location				
Distal	142	63	79	0.275
Proximal	59	21	38	
Differentiation				
High or moderately	53	27	26	0.144
Poor	148	57	91	
Depth of invasion				
T1 + T2	63	56	7	< 0.01
T3 + T4	138	28	110	
Lymph node metastasis				
N0	72	57	15	< 0.01
N _x	129	27	102	
TNM stage				
I-II	91	71	20	< 0.01
III-IV	110	13	97	
Cancerous node				
NO	183	84	99	< 0.01
Yes	18	0	18	
Vascular invasion				
NO	129	72	57	< 0.01
Yes	72	12	60	
Nerve invasion				
NO	119	49	70	0.885
Yes	82	35	47	

that compared to patients with low methylation level of TWA1, patients with higher methylation level of TWA1 had longer OS (Fig. 3G), indicating that the methylation level of TWA1 may be recognized as a biomarker in GC.

3.5. TWA1 mutation in human GC

From the Fig. 4A, we can clearly know the pie chart revealed that the mutant types of GC were all missense substitution. The mutation in GC data contains C>G, G>A, T>G, each of them accounting for 33.33% of the TWA1 coding strand (Fig. 4A). From the cBioportal dataset, we can find that the somatic mutation frequency of TWA1 was only 0.6%.

Table 2
Univariate and multivariate analysis of the association of prognosis with clinicopathological parameters and expression of TWA1 in GC.

Variable	Univariable analysis		Multivariable analysis	
	HR (95%CI)	P-value	HR (95%CI)	P-value
Sex (male vs. female)	0.832 (0.594–1.166)	0.286	NA	NA
Age (< 60 vs. ≥ 60 years)	0.765 (0.562–1.040)	0.088	NA	NA
Tumor size (< 4 vs ≥ 4 cm)	2.452 (1.794–3.352)	< 0.001	1.183 (0.837–1.673)	0.342
Tumor location (distal vs. proximal)	1.962 (1.414–2.722)	< 0.01	1.385 (0.982–1.952)	0.063
Differentiation (high/moderate vs. poor/none)	1.098 (0.779–1.548)	0.593	NA	NA
Depth of invasion (T1-T2 vs. T3-T4)	10.455 (6.316–17.306)	< 0.001	4.966 (2.538–9.717)	< 0.01
Lymph node metastasis (N ₀ vs N _x)	5.246 (3.623–7.597)	< 0.001	1.239 (0.654–2.344)	0.511
TNM stag (I-II/III-IV)	68.223 (30.079–154.735)	< 0.001	31.842 (11.633–87.153)	< 0.001
Cancerous node (NO vs Yes)	5.505 (3.253–9.315)	< 0.01	1.902 (1.100–3.287)	0.021
Vascular invasion (NO vs. Yes)	2.830 (2.049–3.908)	< 0.001	1.330 (0.933–1.895)	0.115
Nerve invasion (NO vs. Yes)	1.178 (0.863–1.608)	0.301	NA	NA
TWA1 expression (low vs. high)	3.792 (2.694–5.339)	< 0.001	1.698(1.053–2.740)	0.03

3.6. Co-expression of TWA1 mRNA

To further investigate the underlying regulation of TWA1 in GC, we searched on the cBioPortal database and found that TATA-box binding protein associated factor 4 (TAF4) was a principal GC gene (Fig. 4B). Regression analysis revealed that TWA1 and TAF4 had high relevant coefficients by cBioPortal (Spearman’s correlation = 0.80; Pearson’s correlation = 0.80) (Fig. 4C). The positive correlation between TWA1 and TAF4 was verified by GEPIA database (Fig. 4D). As a member of transcription factor, TAF4 may bind to the promoter of TWA1 and regulate its transcription. The ChIPbase, which is an open database for studying the transcription factor binding sites and motifs [20], also showed the similar close relationship between TWA1 and TAF4 no matter in TCGA STAD or GTEx stomach (Fig. 4E and F), indicated that TAF4 may bind to TWA1 and regulate its expression. While, all of the guesses need further experimental verification.

3.7. TAF4 mRNA expression and prognosis in patients with GC

To further investigate the expression of TAF4, the GEPIA database was searched. The results showed that TAF4 was overexpressed in GC tissues compared to the normal gastric tissues (Fig. 4G). To evaluate the relationship between TAF4 and prognosis of GC patients, we conducted Kaplan-Meier [21], which include 1065 GC patients with relapse-free and overall survival data, survival analysis to determine survival according to the expression level of TAF4. It was determined that a high expression of TAF4 mRNA was significantly associated with poorer overall survival (OS) and post-progression survival (PPS) (Fig. 4H and I).

Then we predicted potential protein interactions with TWA1 by string database [22]. The results indicated that TWA1 may interact with GID4, WRD26, ARMC8, RMND5B, SPRYD3, RMND5A, RANBP10, MKLN1, MPHOSPH8 and RANBP9 (Fig. 4J).

4. Discussion

Though great advancements in the treatments for GC have been acquired in recent years, the 5-year survival rate of patients diagnosed with advanced GC is still poor [3]. Therefore, finding an effective molecular markers and illuminating the underlying molecular mechanism for clinical diagnosis and therapy is very urgent and necessary.

TWA1 had been reported to function as an oncogene in CRC tissues, its deficiency not only decreases β-catenin nuclear levels and Wnt target gene expression in CRC cells, but also inhibits the proliferation and tumorigenicity of CRC cells in nude mice [9], however, its role in other types of tumor are still unknown. In the present study, we revealed that TWA1 was significantly overexpressed in GC tissues compared with that in the adjacent normal tissues for the first time. As far as we know,

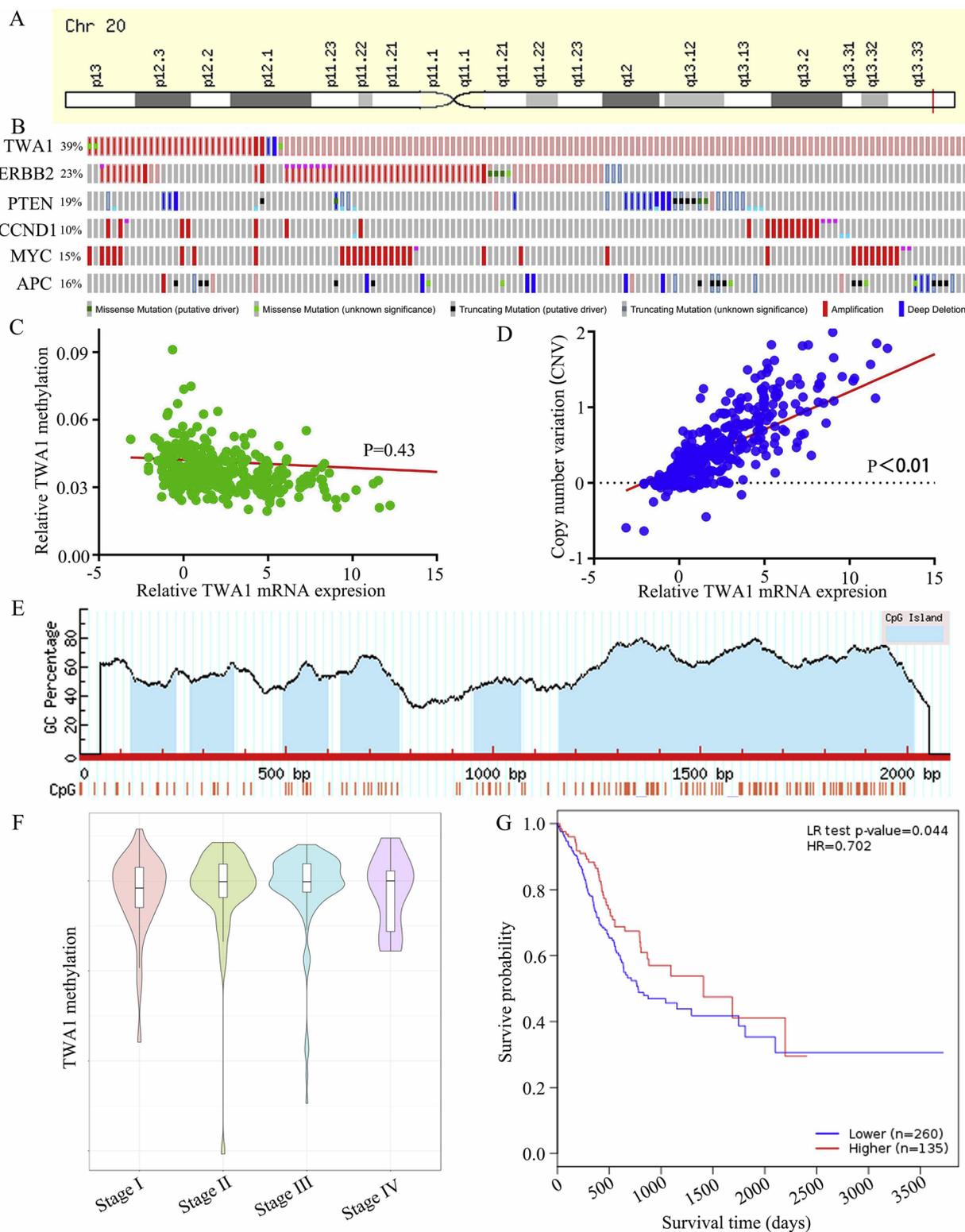


Fig. 3. DNA copy number gain contributes to TWA1 overexpression and promoter hypermethylation of TWA1 predicts favorable prognosis. **A.** TWA1 resides on the long arm of chromosome 20 (20q13.33). **B.** TWA1 present overexpression in GC with amplification and mRNA upregulation compared to ERBB2, PTEN, CCND1, MYC and APC. **C.** There is no significant association between TWA1 mRNA expression and DNA methylation ($P = 0.43$) (the data download from the cBioPortal database). **D.** TWA1 mRNA expression was positively correlated with DNA copy number gain in GC tissues ($P < 0.01$) (the data download from the cBioPortal database). **E.** MethPrimer analysis presented there are many CpG islands in the promoter of TWA1. **F.** MethSurv analysis showed the methylation level of TWA1 is not different in diverse TNM stage. **G.** Patients with higher methylation level of TWA1 had longer OS compared to patients with low methylation level of TWA1 analyzed by MethSurv ($P = 0.044$).

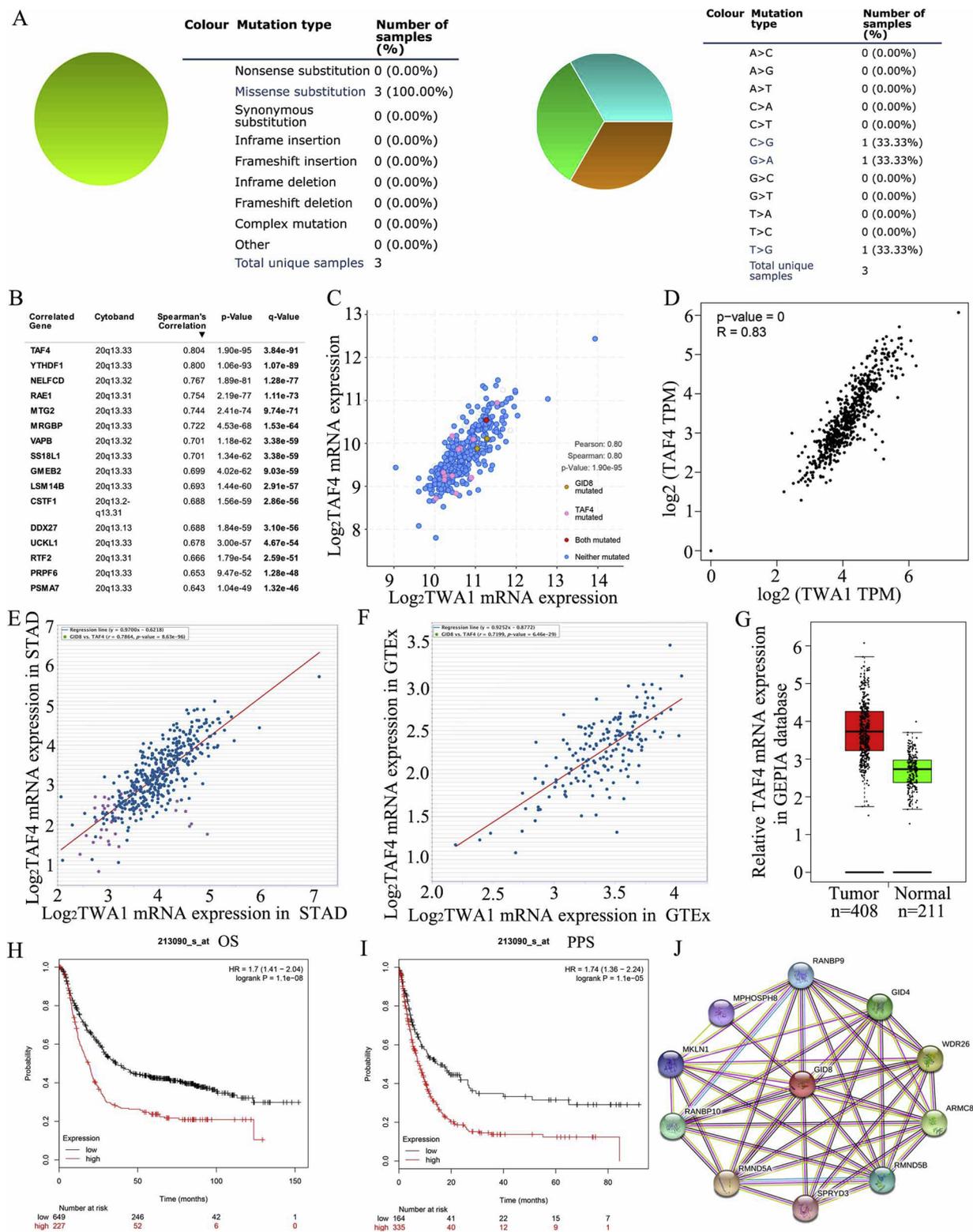


Fig. 4. Co-expression of TWA1 mRNA and TAF4 mRNA, which is associated with adverse prognosis in patients with GC. A. The mutation of TWA1 in GC analyzed by Cosmic database. B. cBioPortal database analyzed the gene co-expression with TWA1. C. Regression analysis revealed that TWA1 and TAF4 had high relevant coefficients by cBioPortal (Spearman's correlation = 0.80; Pearson's correlation = 0.80). D. The positive correlation between TWA1 and TAF4 was verified by GEPIA database (R = 0.83, $P < 0.05$). E. The relationship between TWA1 and TAF4 in TCGA STAD analyzed by ChIPbase ($r = 0.7864$, $P = 8.63e-96$). F. The relationship between TWA1 and TAF4 in GTEx stomach analyzed by ChIPbase ($r = 0.7199$, $P = 6.46e-29$). G. Relative expression of TAF4 mRNA in human GC tissues (Tumor, $n = 408$) and gastric normal tissues (Normal, $n = 211$) from GEPIA database. H. Kaplan-Meier plotter survival analysis (<http://kmplot.com/analysis/>) showed TAF4 mRNA was significantly associated with poorer OS (HR = 1.7 (1.41–2.04); $P = 1.1e-08$). I. Kaplan-Meier plotter survival analysis (<http://kmplot.com/analysis/>) showed TAF4 mRNA was significantly associated with poorer PPS (HR = 1.74 (1.36–2.24); $P = 1.1e-05$). J. The STRING database predicted potential protein interact with TWA1.

Helicobacter pylori infection is recognized as a major risk factor for GC progression [23,24] and EB virus, which display recurrent PIK3CA mutations and extreme DNA hypermethylation, is a pathogenic virus associated with gastric [25,26]. However, bioinformatic analysis study found TWA1 overexpressed in GC is not associated with EB virus infection and HP infection. Overexpression of TWA1 was not only found in GC, but also found in CHOL, COAD, LIHC, READ and LUAD, indicated that TWA1 may play an important role in tumor progression.

To illuminate the role of TWA1 in GC, IHC was used to determinate its expression in 201 primary GC tumor tissues and 60 adjacent non-tumor tissues. The results of IHC showed that TWA1 protein was present in both the cytoplasm and nucleus, but mainly in the cytoplasm, which was further verified by Tissue IF. High expression of TWA1 mRNA and protein were also found in GC tissues by qRT-PCR and Western Blot. The high expression level of TWA1 was also associated with tumor size, depth of invasion, lymph node metastasis, TNM stage, cancerous node and vascular invasion. However, we didn't find significant difference of TWA1 expression in GC with different pathological differentiation degrees, which is not inconsistent with the previous study [27]. Furthermore, higher TWA1 expression was also found to be significantly associated with shorter PFS and OS in GC. The univariate and multivariate analysis of 201 patients with GC in the present study suggested the expression of TWA1 was an independent factor of prognosis in GC. All of the results suggested that the high expression of TWA1 was correlated with unfavorable prognosis of GC patients.

Considering that overexpression of TWA1 is associated with clinicopathology and poor prognosis, it is reasonable to investigate the underlying mechanism. As far as we known, CNA gain and DNA methylation are the two main reasons for gene mRNA overexpression [28,29]. After analyzed the TCGA gastric data, we found that overexpression of TWA1 in GC is mainly due to copy number variation, not DNA methylation. These findings are consistent with the previous studies that genes located on the 20q chromosome have a high frequency (63%) of copy number amplification and TWA1 present amplification and gain at 20q13.33 [27,29]. However, the promoter region of TWA1 presented many CpG islands, which means TWA1 has a great potential to be methylated. Further analysis revealed the promoter methylation of TWA1 didn't influence its expression, which means there are many other mechanisms influence TWA1 expression. Lower methylation of TWA1 was associated with poor prognosis, implicating the potential malignant role of promoter methylation of TWA1 in GC. However, we should also note that DNA methylation levels do not affect TWA1 expression, but affect patient prognosis, which may be only statistically significant. All of the results were based on bioinformatics analysis, whether these results can be replicated in GC tissues and cells which needs to be further verified in future studies.

To further illuminate the regulation mechanism of TWA1, the co-expression and correlation data was searched and found TAF4 was the optimal gene. As one of subunits of TFIID, TAF4 plays an important role in gene transcription, cell cycle, cell proliferation, differentiation and migration [30,31]. It was also recognized as a new strategy for cell reprogramming by targeting alternative splicing of TAF4 [32]. Thus, the mRNA expression of TAF4 was analyzed by RNA sequencing expression data in GC and the results showed TAF4 mRNA was significantly upregulated in GC. The survival results also revealed that high expression of TAF4 mRNA was associated with adverse OS and PPS. All of the results remind us TAF4 may be a valuable biomarker for the prognosis of patients with GC. At the same time, we also noticed that TAF4 is a member of the transcription factor (TF) and involved in interactions with cellular transcriptional activators [33]. The ChIPbase database also suggests that TWA1 have binding sites with TAF4. These results indicated that TAF4 may function as a TF regulate TWA1 expression, which further to mediate tumor invasion and metastasis.

The results of STRING remind us TWA1 may interact with many important proteins related to cancer, including RANBP9, ARMC8, WAD26 and so on. RANBP9 affects cancer cells response to genotoxic

stress and its overexpression is associated with worse response to platinum in NSCLC patients [34]. ARMC8 plays an important role in regulating cell migration, proliferation, tissue maintenance, signal transduction, and tumorigenesis, including osteosarcoma, hepatocellular carcinoma, colon cancer and so on [35,36]. All of the results stimulate us to search further for an efficient target of TWA1 and explore for more specific mechanism of TWA1 in GC.

5. Conclusion

In summary, our study revealed that increased expression of TWA1 may be associated with GC progression. High TWA1 expression was significantly correlated with a poorer OS in patients with GC. Further exploration of the specific mechanism of TWA1 in GC is of great potential import, which need future experimental verification. These findings are of potential clinical utility and merit further validation.

Declaration of Competing Interest

All of the authors declared that there is no conflict of interest.

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References

- [1] E. Van Cutsem, X. Sagaert, B. Topal, K. Haustermans, H. Prenen, Gastric cancer, *Lancet* (London, England) 388 (2016) 2654–2664.
- [2] J. Ferlay, I. Soerjomataram, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, D.M. Parkin, D. Forman, F. Bray, Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012, *Int. J. Cancer* 136 (2015) E359–386.
- [3] C. Allemani, T. Matsuda, V. Di Carlo, R. Harewood, M. Matz, M. Niksic, A. Bonaventure, M. Valkov, C.J. Johnson, J. Esteve, O.J. Ogunbiyi, E.S.G. Azevedo, W.Q. Chen, S. Eser, G. Engholm, C.A. Stiller, A. Monnereau, R.R. Woods, O. Visser, G.H. Lim, J. Aitken, H.K. Weir, M.P. Coleman, Global surveillance of trends in cancer survival 2000–14 (CONCORD-3): analysis of individual records for 37 513 025 patients diagnosed with one of 18 cancers from 322 population-based registries in 71 countries, *Lancet* (London, England) 391 (2018) 1023–1075.
- [4] R.L. Siegel, K.D. Miller, A. Jemal, *Cancer Statistics, 2017*, *CA Cancer J. Clin.* 67 (2017) 7–30.
- [5] M. Umeda, H. Nishitani, T. Nishimoto, A novel nuclear protein, Twa1, and Muskelin comprise a complex with RanBPM, *Gene* 303 (2003) 47–54.
- [6] T.S. de Araujo, M.S. Almeida, (1)H, (13)C and (15)N chemical shift assignment of lissencephaly-1 homology (LisH) domain homodimer of human two-hybrid-associated protein 1 with RanBPM (Twa1), *Biomol. NMR Assign.* 12 (2018) 99–102.
- [7] O. Francis, G.E. Baker, P.R. Race, J.C. Adams, Studies of recombinant TWA1 reveal constitutive dimerization, *Biosci. Rep.* 37 (2017).
- [8] O. Francis, F. Han, J.C. Adams, Molecular phylogeny of a RING E3 ubiquitin ligase, conserved in eukaryotic cells and dominated by homologous components, the muskelin/RanBPM/CTLH complex, *PLoS One* 8 (2013) e75217.
- [9] Y. Lu, S. Xie, W. Zhang, C. Zhang, C. Gao, Q. Sun, Y. Cai, Z. Xu, M. Xiao, Y. Xu, X. Huang, X. Wu, W. Liu, F. Wang, Y. Kang, T. Zhou, Twa1/Gid8 is a beta-catenin nuclear retention factor in Wnt signaling and colorectal tumorigenesis, *Cell Res.* 27 (2017) 1422–1440.
- [10] Z. Fang, L. Zhang, Q. Liao, Y. Wang, F. Yu, M. Feng, X. Xiang, J. Xiong, Regulation of TRIM24 by miR-511 modulates cell proliferation in gastric cancer, *J. Exp. Clin. Cancer Res.* CR 36 (2017) 17.
- [11] S.A. Forbes, D. Beare, H. Boutselakis, S. Bamford, N. Bindal, J. Tate, C.G. Cole, S. Ward, E. Dawson, L. Ponting, R. Stefancsik, B. Harsha, C.Y. Kok, M. Jia, H. Jubb, Z. Sondka, S. Thompson, T. De, P.J. Campbell, COSMIC: somatic cancer genetics at high-resolution, *Nucleic Acids Res.* 45 (2017) D777–d783.
- [12] Z. Tang, C. Li, B. Kang, G. Gao, C. Li, Z. Zhang, GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses, *Nucleic Acids Res.* 45 (2017) W98–w102.
- [13] D.R. Rhodes, S. Kalyana-Sundaram, V. Mahavisno, R. Varambally, J. Yu, B.B. Briggs, T.R. Barrette, M.J. Anstet, C. Kincaid-Beal, P. Kulkarni, S. Varambally, D. Ghosh, A.M. Chinnaiyan, OncoPrint 3.0: genes, pathways, and networks in a collection of 18,000 cancer gene expression profiles, *Neoplasia* (New York, N.Y.) 9 (2007) 166–180.
- [14] D.S. Chandrashekar, B. Bashel, S.A.H. Balasubramanya, C.J. Creighton, I. Ponce-Rodriguez, B. Chakravarthi, S. Varambally, UALCAN: a portal for facilitating tumor

- subgroup gene expression and survival analyses, *Neoplasia* (New York, N.Y.) 19 (2017) 649–658.
- [15] T. Li, J. Fan, B. Wang, N. Traugh, Q. Chen, J.S. Liu, B. Li, X.S. Liu, TIMER: a web server for comprehensive analysis of tumor-infiltrating immune cells, *Cancer Res.* 77 (2017) e108–e110.
- [16] L.C. Li, R. Dahiya, MethPrimer: designing primers for methylation PCRs, *Bioinformatics* (Oxford, England) 18 (2002) 1427–1431.
- [17] K. Yamashita, K. Hosoda, N. Nishizawa, H. Katoh, M. Watanabe, Epigenetic biomarkers of promoter DNA methylation in the new era of cancer treatment, *Cancer Sci.* 109 (2018) 3695–3706.
- [18] G. Liang, D.J. Weisenberger, DNA methylation aberrancies as a guide for surveillance and treatment of human cancers, *Epigenetics* 12 (2017) 416–432.
- [19] V. Modhukur, T. Iljasenko, T. Metsalu, K. Lokk, T. Laisk-Podar, J. Vilo, MethSurv: a web tool to perform multivariable survival analysis using DNA methylation data, *Epigenomics* 10 (2018) 277–288.
- [20] K.R. Zhou, S. Liu, W.J. Sun, L.L. Zheng, H. Zhou, J.H. Yang, L.H. Qu, ChIPBase v2.0: decoding transcriptional regulatory networks of non-coding RNAs and protein-coding genes from ChIP-seq data, *Nucleic Acids Res.* 45 (2017) D43–d50.
- [21] A.M. Szasz, A. Lanczky, A. Nagy, S. Forster, K. Hark, J.E. Green, A. Boussioutas, R. Busuttill, A. Szabo, B. Gyorffy, Cross-validation of survival associated biomarkers in gastric cancer using transcriptomic data of 1,065 patients, *Oncotarget* 7 (2016) 49322–49333.
- [22] D. Szklarczyk, J.H. Morris, H. Cook, M. Kuhn, S. Wyder, M. Simonovic, A. Santos, N.T. Doncheva, A. Roth, P. Bork, L.J. Jensen, C. von Mering, The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible, *Nucleic Acids Res.* 45 (2017) D362–d368.
- [23] Y. Song, G. Liu, S. Liu, R. Chen, N. Wang, Z. Liu, X. Zhang, Z. Xiao, L. Liu, *Helicobacter pylori* upregulates TRPC6 via Wnt/beta-catenin signaling to promote gastric cancer migration and invasion, *Onco. Ther.* 12 (2019) 5269–5279.
- [24] A.K. Kamboj, T.G. Cotter, A.S. Oxentenko, *Helicobacter Pylori*: the past, present, and future in management, *Mayo Clin. Proc.* 92 (2017) 599–604.
- [25] N. Cancer Genome Atlas Research, Comprehensive molecular characterization of gastric adenocarcinoma, *Nature* 513 (2014) 202–209.
- [26] H. Zhou, S. Tan, H. Li, X. Lin, Expression and significance of EBV, ARID1A and PIK3CA in gastric carcinoma, *Mol. Med. Rep.* 19 (2019) 2125–2136.
- [27] L. Cheng, P. Wang, S. Yang, Y. Yang, Q. Zhang, W. Zhang, H. Xiao, H. Gao, Q. Zhang, Identification of genes with a correlation between copy number and expression in gastric cancer, *BMC Med. Genomics* 5 (2012) 14.
- [28] S. Cho, H.S. Kim, M.A. Zeiger, C.B. Umbricht, L.M. Cope, Measuring DNA copy number variation using high-density methylation microarrays, *J. Comput. Biol.* 26 (2019) 295–304.
- [29] L. Liang, J.Y. Fang, J. Xu, Gastric cancer and gene copy number variation: emerging cancer drivers for targeted therapy, *Oncogene* 35 (2016) 1475–1482.
- [30] J. Kazantseva, K. Palm, Diversity in TAF proteomics: consequences for cellular differentiation and migration, *Int. J. Mol. Sci.* 15 (2014) 16680–16697.
- [31] I. Davidson, D. Kobi, A. Fadloun, G. Mengus, New insights into TAFs as regulators of cell cycle and signaling pathways, *Cell Cycle* (Georgetown, Tex.) 4 (2005) 1486–1490.
- [32] J. Kazantseva, H. Sadam, T. Neuman, K. Palm, Targeted alternative splicing of TAF4: a new strategy for cell reprogramming, *Sci. Rep.* 6 (2016) 30852.
- [33] E. Hibino, R. Inoue, M. Sugiyama, J. Kuwahara, K. Matsuzaki, M. Hoshino, Identification of heteromolecular binding sites in transcription factors Sp1 and TAF4 using high-resolution nuclear magnetic resonance spectroscopy, *Protein Sci.* 26 (2017) 2280–2290.
- [34] A. Tessari, K. Parbhoo, M. Pawlikowski, M. Fassan, E. Rulli, C. Foray, A. Fabbri, V. Embrione, M. Ganzinelli, M. Capece, M.J. Campbell, M. Broggin, K. La Perle, G. Farina, S. Cole, M. Marabese, M. Hernandez, J.M. Amann, G. Pruneri, D.P. Carbone, M.C. Garassino, C.M. Croce, D. Palmieri, V. Coppola, RANBP9 affects cancer cells response to genotoxic stress and its overexpression is associated with worse response to platinum in NSCLC patients, *Oncogene* 37 (2018) 6463–6476.
- [35] D. Zhou, W. Zhang, Y. Wang, L. Chen, J. Luan, ARMC8: a potential diagnostic and therapeutic target for cancers, *Hum. Pathol.* 54 (2016) 201.
- [36] F. Jiang, Y. Shi, H. Lu, G. Li, Armadillo repeat-containing protein 8 (ARMC8) silencing inhibits proliferation and invasion in osteosarcoma cells, *Oncol. Res.* 24 (2016) 381–389.
- [37] H. Li, W. Zhang, M. Yan, J. Qiu, J. Chen, X. Sun, X. Chen, L. Song, Y. Zhang, Nucleolar and spindle associated protein 1 promotes metastasis of cervical carcinoma cells by activating Wnt/beta-catenin signaling, *J. Exp. Clin. Cancer Res.* 38 (2019) 33.
- [38] Y. Lu, S. Xie, W. Zhang, C. Zhang, C. Gao, Q. Sun, Y. Cai, Z. Xu, M. Xiao, Y. Xu, X. Huang, X. Wu, W. Liu, F. Wang, Y. Kang, T. Zhou, Twa1/Gid8 is a beta-catenin nuclear retention factor in Wnt signaling and colorectal tumorigenesis, *Cell Res.* 27 (2017) 1422–1440.