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YEATS Domain Containing 4 Promotes Gastric Cancer Cell Proliferation and Mediates Tumor Progression via Activating the Wnt/β-Catenin Signaling Pathway

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Increased expression of YEATS domain containing 4 (YEATS4) has been reported to have a correlation with progression in many types of cancer. However, the mechanism by which it promotes the development of gastric cancer (GC) is rarely reported. This study aimed to investigate the effect of YEATS4 on cell proliferation and tumor progression. The mRNA and protein expressions of YEATS4 in GC tissues and cell lines were analyzed. BGC-823 cells then overexpressed or silenced YEATS4 by transfection of different plasmids. The regulatory effect of YEATS on cell viability, colony formation, cell apoptosis, and tumor growth in vivo was evaluated. Finally, we explored the underlying regulatory mechanism of YEATS4 on the Wnt/β-catenin pathway. YEATS4 was highly expressed in GC tissues and cell lines. Furthermore, Kaplan–Meier survival analysis and qRT-PCR analysis showed that the increased expression of YEATS4 indicated poor prognosis and tumor progression. The overexpression of YEATS4 significantly promoted cell proliferation and inhibited cell apoptosis, whereas the opposite trends were found upon the downregulation of YEATS4. Western blot analysis showed that the downregulation of YEATS4 inhibited protein expression and phosphorylation of β-catenin. In addition, decreased expressions of c-Myc, CDK6, CDK4, cyclin D1, and Bcl-2 and increased expression of Bax were observed in YEATS4 knockdown cells. Our results showed that increased expression of YEATS4 might play a critical role in promoting GC cell proliferation and apoptosis by activating the Wnt/β-catenin signaling pathway, indicating that the control of YEATS4 expression might be used as a promising therapy for GC.

Key words: YEATS domain containing 4 (YEATS4); Gastric cancer (GC); Proliferation; Apoptosis; Wnt/β-catenin pathway

INTRODUCTION

Gastric cancer (GC), a common digestive tract malignancy, is the leading cause of cancer-related deaths around the world[1,2]. GC usually has a poor prognosis, which is often diagnosed at an advanced stage. The relative 5-year survival rates for GC are below 30% in many countries[3]. Increasing evidence has shown that the occurrence of GC has a relationship with chronic gastritis, gastric polyps, gastric ulcer, diet, environment, and inheritance. Surgical technique and chemotherapy have the main potential as curative therapy for GC. However, these often do not reach the expected results because of the fact that a high proportion of tumors are diagnosed at an advanced stage. The main reasons for the high mortality of GC are cell infiltration and metastasis. Thus, a good understanding of GC molecular mechanisms is desperately needed to find a useful therapeutic method for GC.

YEATS domain containing 4 (YEATS4), which belongs to the YEATS domain family, was originally discovered in human glioma[4]. The name "YEATS" domain is derived from five proteins (Yaf9, ENL, AF9, Taf14, and Sas5)[5]. The YEATS domain has an average length of 83 amino acids and can be found in over 100 proteins from more than 50 organisms. YEATS4 is a highly conserved domain and is found to participate in chromatin modification and transcriptional regulation[6,7]. As an oncogene, the pathogenesis of YEATS4 has been widely investigated in different types of malignant tumors, including astrocytoma, non-small cell lung cancer, and glioblastoma[8,9].

To date, only a few studies about YEATS4 have been available, and the effect of YEATS4 on GC has rarely been reported. In the present study, YEATS4 expression in GC tissues and nontumor tissues was investigated. We discovered that YEATS4 was highly expressed in GC.

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Furthermore, YEATS4 regulated cell proliferation via activation of the Wnt/β-catenin signaling pathway. In addition, YEATS4 inhibited apoptosis via the upregulation of Bcl-2. Our study demonstrated the function and mechanism of YEATS4 in GC, which provided potential therapeutic targets for the treatment of GC.

MATERIALS AND METHODS

Cell Culture

Human GC cell lines BGC-823, MKN-45, AGS, SGC-7901, MKN-28, and MGC-803 and the human gastric mucosal epithelial cell line GES-1 were purchased from the Cell Bank of Institute of Cell Biology (Shanghai, P.R. China). The cells were cultured in RPMI-1640 culture medium (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and maintained at 37°C in 95% air and 5% CO_2.

Cell Transfections

Full-length human YEATS4 was amplified and inserted into pcDNA3.1 to construct the YEATS4-overexpressed vector (pc-YEATS4). Cell transfection was conducted using Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's instructions. The pSiCoR vector carrying short hairpin RNA (shRNA) against YEATS4 (shYEATS4) was synthesized by GenePharma (Shanghai, P.R. China), packaged into lentivirus, and infected into cells to silence YEATS4 in line with the manufacturer's protocol. Cells transfected with pcDNA3.1 or the pSiCoR vector containing nontargeting shRNA sequence (shNC) were considered to be negative controls.

MTT Assay

The cell proliferative capacity was determined using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay according to standard methods described previously. Each experiment was carried out three times.

Quantitative RT-PCR Analysis

Total RNA was extracted from transfected cells using TRIzol reagent (Invitrogen) and DNase I (Promega, Madison, WI, USA). Reverse transcription was performed using the Multiscribe RT Kit (Applied Biosystems, Foster City, CA, USA) and random hexamers or oligo (dT). Subsequent quantitative PCR (qPCR) was performed using Power SYBR Green Master Mix (Applied Biosystems) in accordance with the supplier's instructions. The mRNA-specific primers for quantitative real-time (qRT-PCR) were as follows: YEATS4, 5′-TCA TAG AAC TCT GAA ACC ACT GTC-3′ (forward) and 5′-TCA TAG AAC TCT GAA ACC ACT GTC-3′ (reverse); GAPDH, 5′-TGA CTT CAA CAG CGA CAC CCA-3′ (forward) and 5′-CAC CCT GTT GCT GTA GCC AAA-3′ (reverse). The reverse transcription conditions were 10 min at 25°C, 30 min at 48°C, and a final step of 5 min at 95°C. The relative expression was calculated with the $2^{-\Delta\Delta Ct}$ method[10]. GAPDH acted as the housekeeping gene.

Western Blot Analysis

The protein used for Western blotting was extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, P.R. China) supplemented with protease inhibitors (Roche, Guangzhou, P.R. China). The proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The Western blot system was established using a Bio-Rad Bis-Tris Gel System according to the manufacturer's instructions. Primary antibodies against YEATS4 (ab205018), GAPDH (ab128915), phosphorylated β-catenin (p-β-catenin, ab138378), β-catenin (ab6302), Bcl-2 (ab32124), Bax (ab77566), c-Myc (ab 152146), CDK6 (ab151247), CDK4 (ab137818), and cyclin D1 (ab137875) (all from Abcam, Cambridge, MA, USA) were prepared in 5% blocking buffer. Primary antibodies were respectively incubated with the membrane at 4°C overnight, followed by wash and incubation with secondary antibodies marked by horseradish peroxidase for 1 h at room temperature. After rinsing, the polyvinylidene difluoride (PVDF) membrane carrying blots and antibodies were transferred into the Bio-Rad ChemiDoc™ XRS System, and then 200 μl of Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) was added to cover the membrane surface.

Evaluation of Tumorigenicity

A total of 2×10^6 BGC-823 cells, respectively transfected with shNC, shYEATS4, or pc-YEATS4, were subcutaneously injected into the flanks of 6-week-old female BALB/c-nu mice ($n=10$ for each group; Animal Center of Nanjing Medical University, P.R. China). The mice were maintained in cages with food and water available ad libitum. All experiments were approved by the ethics committee of Nanjing Medical University. The tumor size was measured after 4 weeks.

Colony Formation Assay

BGC-823 cells were digested into single-cell suspension and then inoculated into six-well plates with 600 cells and a final volume of 2 ml of culture medium per well. The cells were then cultured at 37°C with 5% CO_2 for 3 weeks, during which time the culture medium was changed every 4 days. At the end of the experiment, the cells were stained by 0.5% crystal violet for 15 min,

and the number of clones was counted under an inverted microscope (Olympus, Tokyo, Japan).

Chromatin Immunoprecipitation (ChIP)

ChIP assay was carried out following the standard instructions (Upstate Biotechnology, Inc.). BGC-823 cells were treated with 1% formaldehyde for 10 min, cracked with SDS lysis buffer and broken by the ultrasonic wave, and then incubated with antibodies against YEATS4 (ab205018) or histone H3 acetylated at lysine 27 (H3K27Ac, ab4729) (both from Abcam). Normal human rabbit IgG was used as a negative control. After washing with high salt, low salt, and LiCl buffer, the eluent was used to harvest the chromatin fragments. Finally, de-crosslinking was performed, and the enrichment was examined using RT-PCR.

Luciferase Activity Assay

The pmirGLO vector (Promega) containing the full-length promoter of β-catenin (pmirGLO-β-catenin) was synthesized by GenePharma. pGL-β-catenin was then transfected into BGC-823 cells alone or with pc-YEATS4 using Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's instructions. The plasma pRL-TK (Promega) containing the *Renilla* gene was also transfected into cells acting as an internal control. After transfection, the luciferase activity was assayed using the Dual-Luciferase Reporter Assay System (Promega) in accordance with the manufacturer's protocol.

Statistical Analysis

All experiments were repeated three times. The results of multiple experiments were presented as the mean ± SD. Statistical analyses were performed using SPSS 19.0 statistical software. The p values were calculated using a one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered to indicate statistically significant results.

RESULTS

YEATS4 Was Highly Expressed in GC Tissues and Cell Lines

qRT-PCR analysis was used to analyze the mRNA expression level of YEATS4 in 30 GC patients, both in tumor tissues and paired adjacent noncancerous regions of the tissues. The results showed that YEATS4 was significantly upregulated in GC tumor tissues compared with adjacent noncancerous gastric tissues ($p < 0.05$) (Fig. 1A). Only about 13% (4/30) of GC patients had a lower expression of YEATS4 in GC tissues relative to the paired adjacent noncancerous tissue (Fig. 1B). In addition, protein expression of YEATS4 was obviously increased in tumor samples compared with the nontumor tissues of four patients (Fig. 1C). Furthermore, an increased

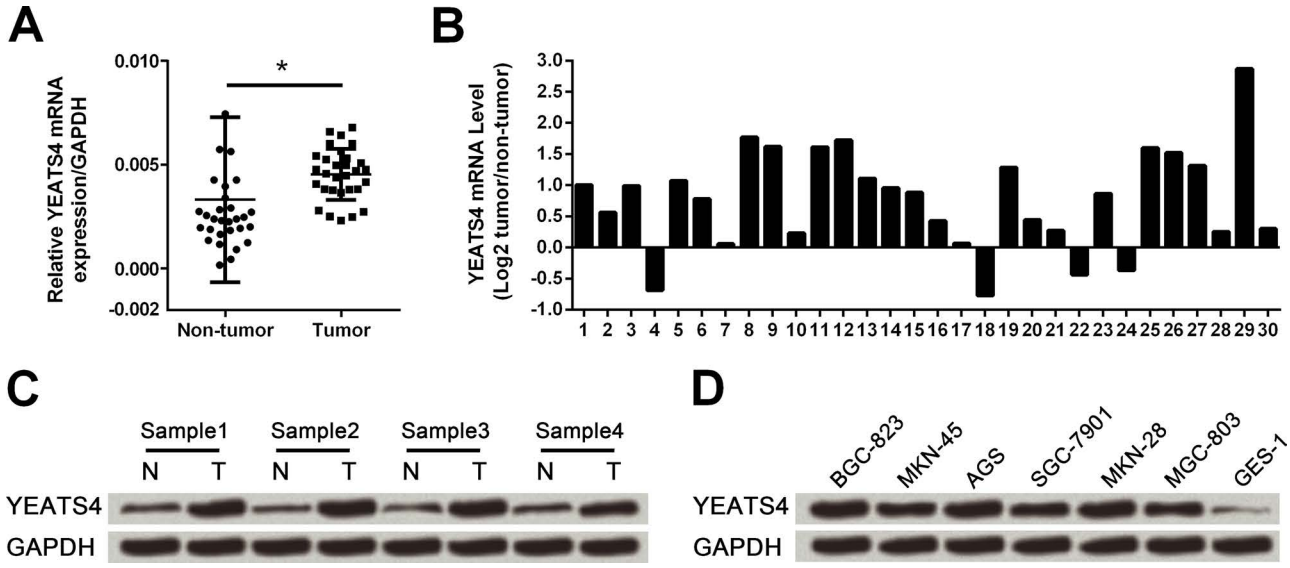


Figure 1. YEATS domain containing 4 (YEATS4) was highly expressed in gastric cancer (GC) tissues and cell lines. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to analyze the mRNA expression levels of YEATS4. (A) YEATS4 was highly expressed in GC tissues. Data are presented as mean ± SD. *$p < 0.05$. (B) YEATS4 mRNA expression levels in 30 paired GC tissues. (C) Protein expression of YEATS4 in GC tissues and paired adjacent noncancerous tissues. (D) The protein expression level of YEATS4 in six GC cell lines and the normal cell line GES-1. GAPDH was used as an internal control. N, nontumor tissues; T, tumor tissues.

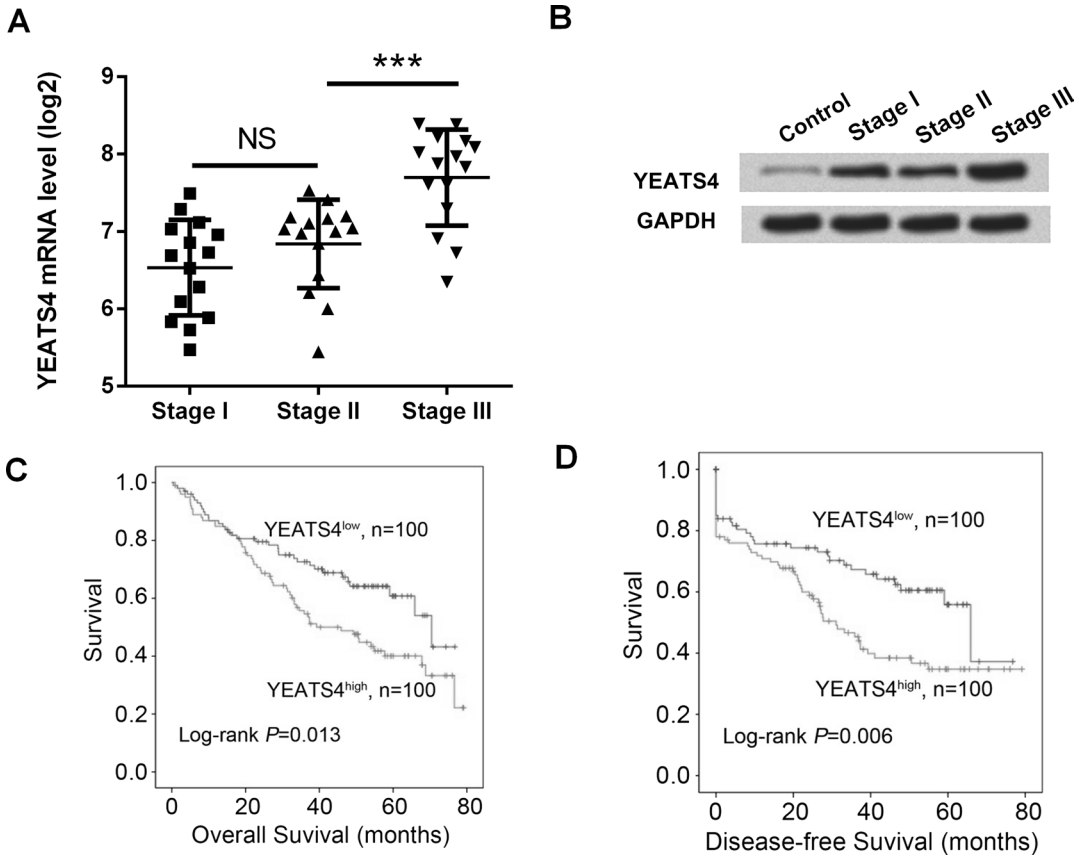


Figure 2. The overexpression of YEATS4 indicated poor prognosis and tumor progression. (A) The mRNA expression of YEATS4 in different stages of GC. Data are presented as mean ± SD. ***$p < 0.001$; $^{NS}p > 0.05$. (B) The protein expression of YEATS4 in different stages of GC. (C) Kaplan–Meier survival analysis of total survival rate. (D) Kaplan–Meier survival analysis of disease-free survival rate.

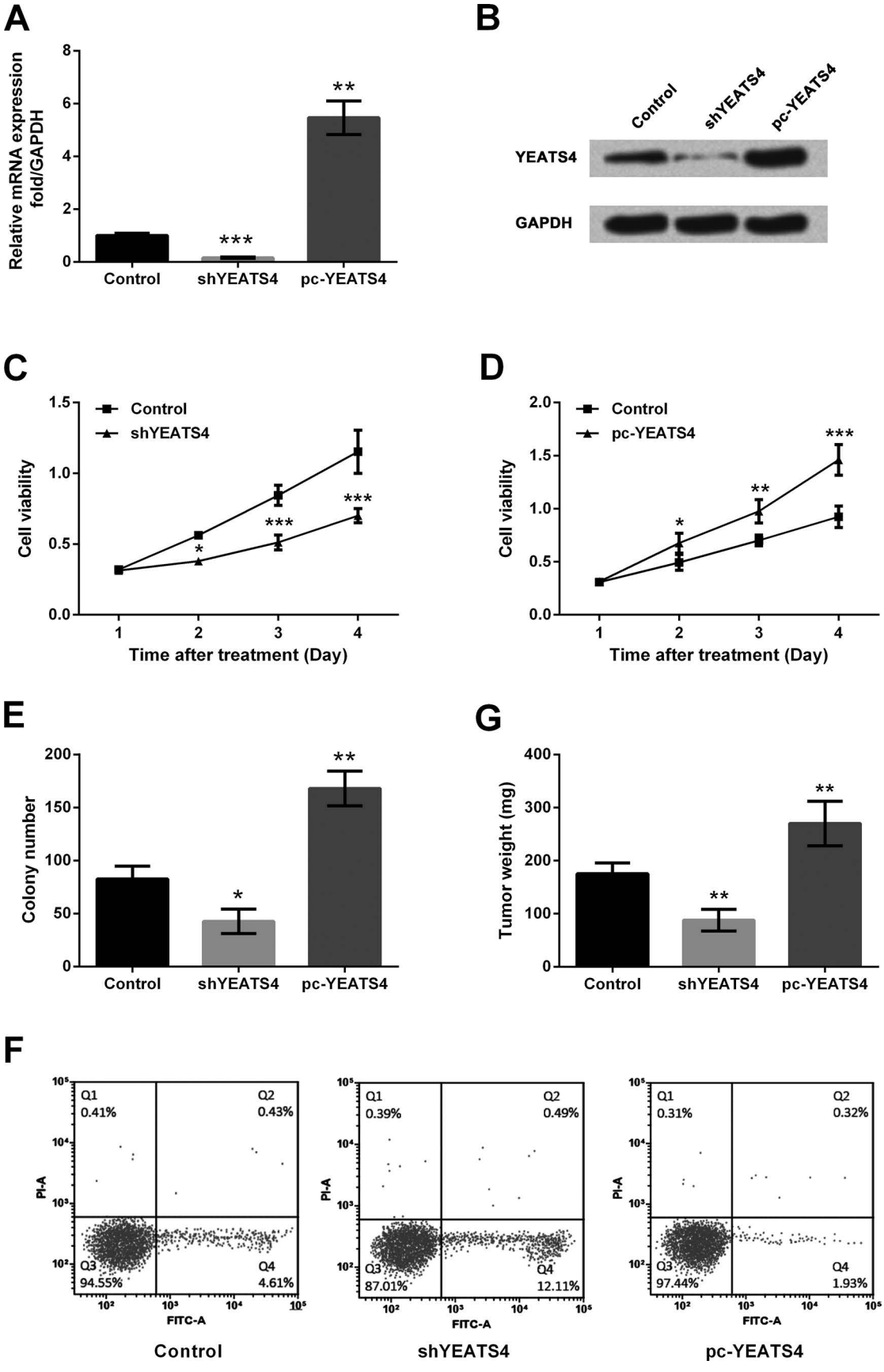
protein expression level of YEATS4 was found in different GC cell lines, including BGC-823, MKN-45, AGS, SGC-7901, MKN-28, and MGC-803, compared with the human gastric mucosal epithelial cell line GES-1, and the YEATS4 expression was highest in BGC-823 among the six GC cell lines (Fig. 1D). Therefore, the BGC-823 cell line was chosen for the following experiments.

Overexpression of YEATS4 Indicated Poor Prognosis and Tumor Progression

qRT-PCR analysis was used to detect the expression level of YEATS4 in different stages of GC tissues. The results showed that the expression level of YEATS4 was the highest in stage III compared with the other two stages. There was no significant statistical difference between stage I and stage II (Fig. 2A). A similar phenomenon was found in the protein expression level of YEATS4 when assessed by Western blot analysis (Fig. 2B). The SPSS survivorship curve was used to analyze the survival rate. A high expression of YEATS4 indicated a low total survival rate (Fig. 2C). Similar results were found in the disease-free survival rate (Fig. 2D), which showed that the overexpression of YEATS4 indicated tumor progression.

FACING PAGE

Figure 3. YEATS4 regulated the cell proliferation and apoptosis of BGC-823 cells. (A) The downregulation and upregulation of YEATS4 in BGC-823 cells. (B) Western blot analysis of the protein expression of YEATS4. (C) shYEATS4 decreased cell viability in BGC-823 cells. (D) pc-YEATS4 increased cell viability in BGC-823 cells. (E) YEATS4 promoted colony formation of BGC-823 cells. (F) YEATS4 inhibited cell apoptosis of BGC-823 cells. (G) The effect of YEATS4 on GC in vivo. Data are presented as mean ± SD. Each experiment was repeated at least three times. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. shYEATS4, pSiCoR vector carrying short hairpin RNA against YEATS4; pc-YEATS4, pcDNA3.1 carrying full-length coding sequence of YEATS4.


A
Relative mRNA expression fold/GAPDH
Control
shYEATS4
pc-YEATS4
B
Control
shYEATS4
pc-YEATS4
YEATS4
GAPDH
C
Control
shYEATS4
Cell viability
Time after treatment (Day)
D
Control
pc-YEATS4
Cell viability
Time after treatment (Day)
E
Colony number
Control
shYEATS4
pc-YEATS4
G
Tumor weight (mg)
Control
shYEATS4
pc-YEATS4
F
Q1 0.41%
Q2 0.43%
Q3 94.55%
Q4 4.61%
Q1 0.39%
Q2 0.49%
Q3 87.01%
Q4 12.11%
Q1 0.31%
Q2 0.32%
Q3 97.44%
Q4 1.93%
PI-A
FITC-A
Control
shYEATS4
pc-YEATS4

YEATS4 Regulated the Proliferation and Apoptosis of BGC-823 Cells

To explore the function of YEATS4 in the progression of GC, BGC-823 cells were transfected with shYEATS4 or pc-YEATS4, respectively. mRNA and protein expression levels of YEATS4 were then assessed by qRT-PCR and Western blot analysis, respectively. When compared to the control group, the expression level of YEATS4 was obviously enhanced in cells transfected with pc-YEATS4 ($p<0.01$) while being significantly decreased in cells infected with shYEATS4 ($p<0.001$) (Fig. 3A and B). Results of the MTT assay showed that cell viability was markedly decreased by YEATS4 knockdown at 2 days ($p<0.05$), 3 days ($p<0.001$), and 4 days ($p<0.001$) after transfection, while it was significantly increased by YEATS4 overexpression at 2 days ($p<0.05$), 3 days ($p<0.01$), and 4 days ($p<0.001$) after transfection when compared to the control group (Fig. 3C and D). A similar effect was observed in the colony formation assay in that the overexpressed YEATS4 cells formed significantly more colonies than the control group ($p<0.01$) (Fig. 3E). Therefore, we concluded that YEATS4 could promote cell proliferation in BGC-823 cells. Furthermore, cell apoptosis using annexin V/PI revealed that downregulation of YEATS4 promoted cell apoptosis in BGC-823 cells, while overexpression of YEATS4 inhibited cell apoptosis (Fig. 3F). To test the effect of YEATS4 on GC in vivo, BGC-823 cells transfected with shYEATS4 or pc-YEATS4 were injected into female nude mice by tail vein injection. Implantation of the BGC-823 cells with overexpressed YEATS4 significantly promoted tumor growth compared to the control group ($p<0.01$), whereas the downregulation of YEATS4 obviously inhibited tumor growth ($p<0.01$) (Fig. 3G).

YEATS4 Activated the Wnt/β-Catenin Signaling Pathway

To better understand the mechanism of the regulatory effect of YEATS4, we explored the difference in activation of the Wnt/β-catenin pathway. In this study, we detected the changes of p-β-catenin, β-catenin, antiapoptotic Bcl-2, proapoptotic Bax, c-Myc, CDK6, CDK4, and cyclin D1 by Western blot analysis. Our results showed that the downregulation of YEATS4 inhibited β-catenin protein expression and phosphorylation of β-catenin (Fig. 4A). However, the opposite trend was seen in cells with overexpression of YEATS4 compared with the control group (Fig. 4B). In addition, YEATS4 knockdown decreased the expression levels of Bcl-2, c-Myc, CDK6, CDK4, and cyclin D1 but increased the expression level of Bax (Fig. 4C). In contrast, the effect of YEATS4 overexpression on protein expression had the opposite effect (Fig. 4D). In order to validate that YEATS4 affected BGC-823 cells through the Wnt/β-catenin pathway, Wnt inhibitor (21H7, sigma, and SML0570) was used to block the Wnt/β-catenin signaling pathway. Results showed that the introduction of 21H7 significantly alleviated the effect of YEATS4 overexpression on the expressions of these proteins, indicating that YEATS4 regulated the expression of these proteins through the Wnt/β-catenin signaling pathway (Fig. 4E). At the same time, Western blot analysis showed that the downregulation of YEATS4 inhibited the expression and phosphorylation of β-catenin in both the nucleus and total cell lysates. The opposite trend was seen in cells with YEATS4 overexpression (Fig. 4F). Decreased expression and phosphorylation of β-catenin were found in GC tissues with downregulation of YEATS4, while the opposite trend was found in GC tissues with overexpression of YEATS4 (Fig. 4G).

YEATS4 Directly Regulated the Transcriptional Expression of β-Catenin

Results of the ChIP assay showed that YEATS4 was deposited in the promoter region of β-catenin in BGC-823 cells (Fig. 5A). The same phenomenon was found in GC tissues (Fig. 5B). In the meantime, more YEATS4 was deposited in the promoter region of β-catenin in GC tissues in comparison with nontumor tissues ($p<0.001$) (Fig. 5C). Furthermore, results of the luciferase assay demonstrated that YEATS4 increased luciferase activity when cotransfected with pGL-β-catenin compared with cells cotransfected with pcDNA3.1 ($p<0.01$), indicating that YEATS4 regulated the transcriptional expression of β-catenin (Fig. 5D). In addition, the enrichment of H3K27Ac was detected in BGC-823 cells. Results showed that the overexpression of YEATS4 obviously promoted the deposition of H3K27Ac, while downregulation of YEATS4 significantly inhibited the deposition of H3K27Ac, indicating that YEATS4 regulates acetylation of the β-catenin promoter region (Fig. 5E).

DISCUSSION

YEATS4, originally isolated from the glioblastoma multiform cell line TX3868, is a highly conserved nuclear protein that plays an important role in cell viability[9,11]. YEATS4 contains 227 amino acids and is frequently amplified in glioblastomas and astrocytomas[12]. For those reasons, YEATS4 is regarded as an oncogene in the development of various cancers. In our study, the expression level of YEATS4 in GC tissues and the effect of YEATS4 on cell proliferation and the Wnt/β-catenin signaling pathway were investigated.

Considering that YEATS4 only contains the DNA activation domain without the DNA binding domain, it is speculated to be a transcription factor with an incomplete structure[13]. The study conducted by Debernardi et al. showed that YEATS4 participated in the progression

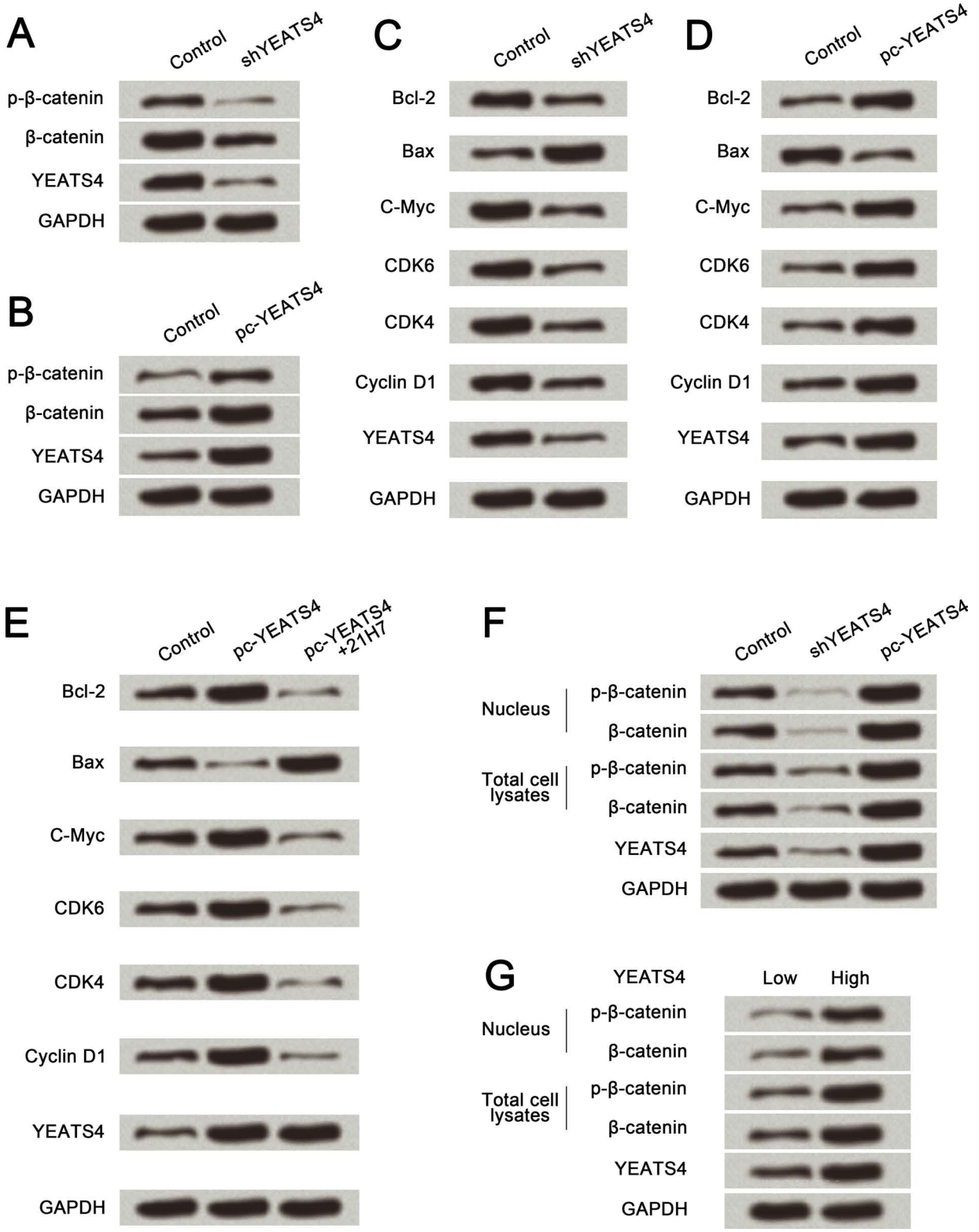


Figure 4. YEATS4 regulated cell proliferation and apoptosis via activation of the Wnt/β-catenin signaling pathway. (A) shYEATS4 downregulated the expression of β-catenin. (B) pc-YEATS4 upregulated the expression of β-catenin. (C) Effect of shYEATS4 on periodic and apoptotic proteins. (D) Effect of pc-YEATS4 on periodic and apoptotic proteins. (E) Effect of Wnt inhibitor on periodic and apoptotic proteins. (F) Expression and phosphorylation of β-catenin in the nucleus and total cell lysates of BGC-823 cells. (G) Expression and phosphorylation of β-catenin in the nucleus and total cell lysates of GC tissues.

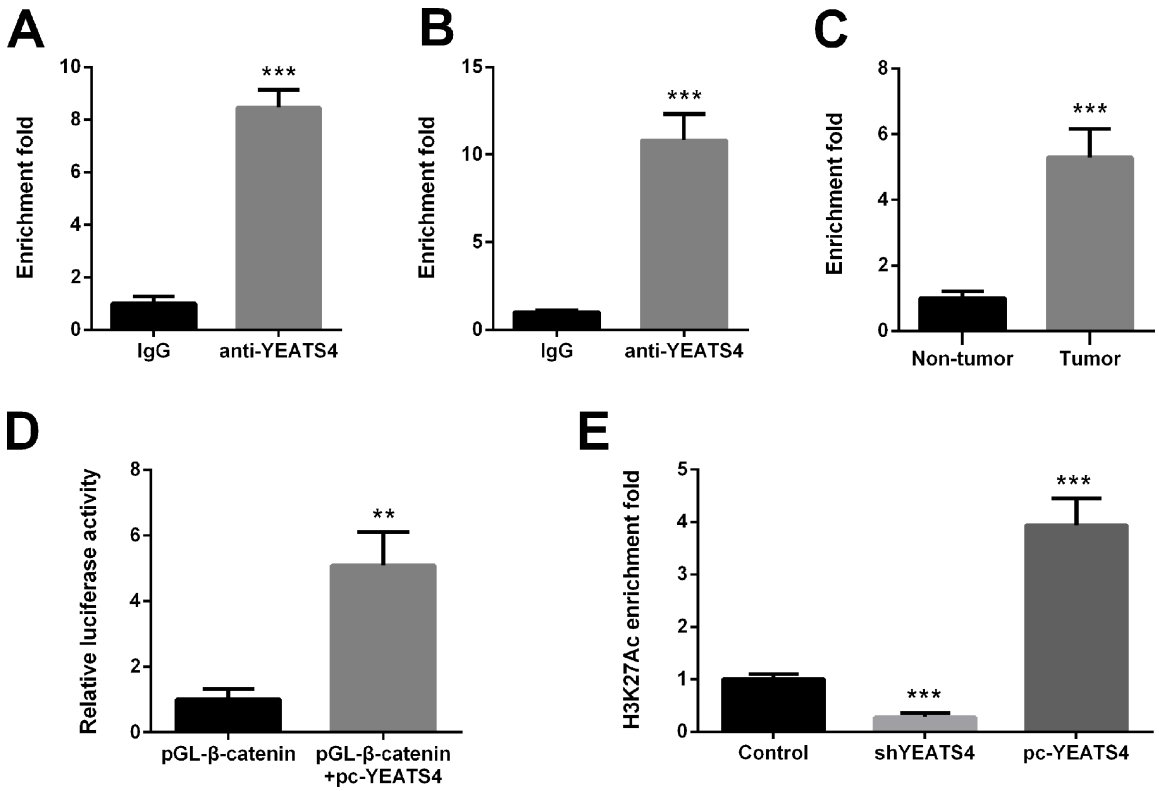


Figure 5. Effect of YEATS4 on the transcriptional expression of β-catenin. (A) YEATS4 deposited in the promoter region of β-catenin in BGC-823 cells. (B) YEATS4 deposited in the promoter region of GC tissues. (C) The deposition of YEATS4 in nontumor and tumor tissues of GC. (D) Luciferase activity. (E) Effect of YEATS4 on H3K27Ac deposition. Data are presented as mean+SD of three independent experiments. $^{**}p<0.01$; $^{***}p<0.001$. pGL-β-catenin, pmir-GLO vector carrying β-catenin promoter; H3K27Ac, histone H3 acetylated at lysine 27.

of leukemia[14]. It had been reported that the downregulation of YEATS4 induced cell death in the chicken prelymphoid cell line DT40, which indicated the critical role of YEATS4 in cell viability[11]. The results of our study showed that YEATS4 was highly expressed in GC tissues and cell lines. In order to better explore the function of YEATS4 in the progression of GC, shYEATS4 and pc-YEATS4 were respectively transfected into BGC-823 cells to downregulate and upregulate the expression of YEATS4. We found that the overexpression of YEATS4 significantly promoted cell proliferation, inhibited cell apoptosis, and promoted tumor growth. A similar effect of YEATS4 was discovered in other studies, in that YEATS4 promoted cell proliferation and inhibited cell apoptosis of lung cancer via the negative regulation of the p21/p53 pathway[8,15].

The Wnt pathway, named from wingless and Int-1, is crucial for cell proliferation, apoptosis, migration, and differentiation. In consideration of the relationship with β-catenin, there are two categories of the Wnt pathway, known as β-catenin-dependent (canonical) or β-catenin-independent (non-canonical) pathways. As a key protein in the Wnt signaling pathway, β-catenin takes part in the development of carcinoma via its accumulation in carcinoma and translocation into the nucleus[16,17]. Translocation of β-catenin into the nucleus induces activation of the canonical Wnt pathway and then interacts with TCF/LEF to cause the transcription of target genes (such as cyclin D1 and c-Myc) in the Wnt/β-catenin signaling pathway[18]. Some studies demonstrated that upregulation of the Wnt-1 ligand had the ability to promote the advanced stages of GC[19,20]. In addition, previous studies found that Wnt-5a promoted cell migration in GC cells via the activation of focal adhesion kinase[21,22]. In our study, we found that YEATS4 regulated the expression of periodic proteins such as cyclin D1, CDK4, CDK6, and c-Myc, and apoptotic proteins such as Bcl-2 and Bax, and YEATS4 regulated the protein expression and phosphorylation of β-catenin in both the nucleus and cell lysates. Additionally, YEATS4 directly regulated the transcriptional expression of β-catenin and regulated acetylation of the β-catenin promoter region. Therefore, we supposed that YEATS4 regulated cell proliferation via activation of the Wnt/β-catenin signaling pathway. However, the mechanism of how YEATS4 affects the Wnt/β-catenin signaling pathway needs to be further explored.

The results of our studies suggest that the increased expression of YEATS4 might be used as a valuable prognostic marker of GC progression through its function of promoting cell proliferation and tumorigenesis and inhibiting cell apoptosis. In addition, YEATS4 regulates cell proliferation via activation of the Wnt/β-catenin signaling pathway, indicating that the control of YEATS4 expression might be used as a promising therapy for GC.

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