YWHAG promotes gastric cancer proliferation and migration via PI3K/AKT pathway

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Research Article

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Abstract

Background: The influence of tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma (YWHAG) remains unknown in gastric cancer (GC). We investigated biological effects of YWHAG on GC and clarified the potential molecular mechanisms.

Methods: YWHAG expression in GC was assessed by immunohistochemistry, followed by correlation analysis. GC cells of YWHAG knockdown and overexpression were constructed by lentivirus transfection. Cell viability of growth was evaluated by cell counting kit-8 (CCK8) and the 5-ethyny-2-deoxyuridine (EdU) assays. Cell cycle and apoptosis were conducted. The following migration and invasion analysis were detected by wound-healing and trans-well assays. The role of YWHAG in vivo was revealed by xenograft models. Gene set enrichment analysis (GSEA) and western blotting were applied for the potential pathways.

Results: YWHAG was elevated in GC (p<0.05), and closely related to differentiation degree. The study demonstrated that YWHAG knockdown significantly inhibited the proliferation, migration, invasion and epithelial-mesenchymal transformation (EMT) of GC cells and decelerated the growth of xenograft tumors, while YWHAG overexpression reversed the effects. Consistent with the results of GSEA, YWHAG activated PI3K/AKT pathway.

Conclusions: YWHAG can promote proliferation and migration of GC, which might be related to the cell cycle, EMT and PI3K/AKT signaling pathway.

Introduction

Gastric cancer (GC) is the fifth most common cancer and the main leading cause of cancer-related death globally[1]. Recently, the incidence and mortality in GC have decreased globally, many patients have experienced postoperative recurrence and chemotherapy resistance[2, 3]. Therefore, exploring therapeutic targets is desperately needed to improve survival of GC patients.

The YWHA (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, 14-3-3) family regulates cell metabolism, signal transduction, and cell cycle apoptosis. It may cause conformational changes in the binding partners by binding to phosphoserine-containing proteins[4]. YWHAG (14-3-3γ), a member of the YWHA protein family, was identified and isolated in 1999[5]. Priors studies showed that YWHAG is overexpressed in malignances such as glioblastoma and breast cancer[5–7]. YWHAG could act as an oncogene in advanced lung cancer and indirectly down-regulated the expression of tumor suppressor p53[7]. YWHAG also inhibited the apoptosis and participated in the metastasis of breast cancer[8, 9]. Mei J et al. reported that miRNA could promote breast cancer cells' epithelial-mesenchymal transformation (EMT) by targeting YWHAG[10]. In conclusion, YWHAG plays different roles in malignant tumors and is a promising molecular target for tumor treatment. However, the effect and mechanisms of YWHAG are unclear in GC.

We investigated the mechanism of YWHAG in GC. The expression pattern of YWAHG in GC tissues was exhibited by immunochemistry (IHC). Next, we constructed stable YWHAG knockdown and overexpression cell lines and further analyzed the biological function of YWHAG in GC cells. A vivo model was applied to verify the in vitro experiment results. In addition, this study illustrated the potential mechanism of YWHAG promoting GC progression. The above findings demonstrated that YWHAG might play an essential role in GC.

Materials And Methods

Clinical specimens

A total of 107 pairs of GC and normal tissues were collected from May 2013 to December 2014 in Lanzhou University Second Hospital. The sample collection was approved by the Ethics Committee of Lanzhou University Second hospital. All patients included in the study did not receive any treatments before surgery.

Immunohistochemistry

Paraffin embedded tissue sections were dewaxed and hydrated. The sections were heated in a 10mM sodium citrate solution at 98°C for antigen retrieval. They were soaked in 0.3% H_2O_2 for 25 min to block endogenous peroxidase. Anti-YWHAG (1:200, Abcam, USA) and anti-Ki67 (1:300, Abcam, USA) were incubated with sections overnight at 4°C. Then sections were stained with diaminobenzidine solution and counterstained with hematoxylin. IHC results were independently evaluated and recorded by two senior pathologists using a blind method. The score was determined based on the percentage of cells stained (0 = 0%-5%, 1 = 6%-25%, 2 = 26%-50%, 3 = 51–75%, 4 = 76% -100%) and staining intensity (0 = no staining,1 = weak staining,2 = moderate staining, and 3 = strong staining). The product of the two scores obtained the final YWHAG and Ki67 scores.

Bioinformatics analysis

The transcription data of GC were obtained from The Cancer Genome Atlas (TCGA) database (https://ancergenome.nih.gov/), which included 375 tumor samples and 32 normal samples. YWHAG gene expression analysis was performed with R software. Gene enrichment analysis (GSEA) software was used to reveal the potential function of YWHAG in GC.

Cell culture

Human GC cell lines MKN45, HGC27, NCI-N87, MKN28, KAO-III, AGS and human normal gastric mucosal epithelial cells (GES-1) were purchased from Cell Culture Center of Chinese Academy of Medical Sciences (Beijing, China). All cell lines were identified by short tandem repeats (STR). They were maintained in RPMI 1640 medium (Gibco, USA) with 10% fetal bovine serum (FBS, Gibco, USA) at 37℃ in a humidified incubator with 5% CO_2.

Lentivirus transfection

YWHAG-overexpression and knockdown lentivirus (GeneChem, Shanghai, China) were transfected into NCI-N87 and MKN45 cell lines. An empty lentivirus vector was used as a negative control. After 72 hours of transfection, the cells were selected with puromycin (Biofroxx, German) for 3 days. The transfection efficiency was detected by Western blot.

Cell counting kit-8 (CCK8) assay

The YWHAG knockdown, overexpression cells and the corresponding negative transfected control cells were seeded in a 96-well plate and cultured in an incubator at 37°C. At the specific time points, 10ul CCK8 solution was added and then incubated for 1 hour. Finally, the absorbance value was measured at the wavelength of 490nm.

EdU assay

Cells were seeded in 96-well plates at a density of 2×10^3 cells per well and incubated with EdU (RiboBio, Guangzhou, China) for 2 hours. After that, 4% paraformaldehyde was used for fixation, and staining was performed according to the reagent manufacturer's instructions. Finally, the Operetta CLS High Content Screening System (PerkinElmer, Waltham, MA).

Cell cycle and apoptosis analysis

Cells were resuspended in 100ul binding buffer, and the cell concentration was adjusted to $2x10^5$ cell/tube. Cells were fixed in 75% ethanol at 4 ° C overnight to evaluate the cell cycle. Then cells were stained with propidium iodide (PI, BD Bioscience, USA). For cell apoptosis, each sample was added with Annexin V-FITC /PI (BD Bioscience, USA) and incubated in the dark at room temperature. Flow cytometry was applied.

Migration assay

The invasion ability of YWHAG on GC was evaluated by an invasion assay of trans-well coated with matrix (BD Bioscience, USA). 5×10^4 cells were resuspended into a serum-free medium and added to the upper chamber. The medium containing 10% FBS was added to the lower chamber as a chemical attractant. The medium was removed after 24 hours, and cells on the membrane surface were wiped clean with a cotton swab. Cells migrated to the lower compartment were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The number of invaded cells was measured under a microscope.

After the cells seeded in a 6-well plate were fused to 90%, a sterile 200ul pipette tip was used to form a scratch wound on the cell monolayer. The cells were washed twice with PBS and cultured in serum-free medium. The width of the wound was measured at 0, 24, 48 and 72 hours.

Western blot

The cells were lysed by radio-immunoprecipitation assay (RIPA) buffer containing protease inhibitor on ice. The same amount of protein was separated by 10%SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane, which was then sealed with blocking solution for 2 hours. Next, the

membranes were incubated with the corresponding primary antibody overnight at 4℃. The primary antibodies used were: anti-GAPDH (1:10000, Proteintech, China), anti-YWHAG (1:1000, Abcam, USA), anti-cyclinD1 (1:1000, Abcam, USA), anti-CDK2 (1:1000, Abcam, USA), anti-E-cadherin (1;1000, Proteintech, China), anti-vimentin (1:2000, Proteintech, China), anti-AKT (1:1000, Abcam, USA), anti-p-AKT (1:1000, Abcam, USA) and anti-p-PI3K (1:1000, Abcam, USA). After incubation with the secondary antibodies at room temperature for 1 hour, the blots were exposed with a chemiluminescent detection system.

Tumor xenograft model

BALB/c nude mice aged 5–6 weeks were purchased from Hangzhou Ziyuan Laboratory Animal Technology Co., Ltd. (Hangzhou, China). All mice were raised in a specific pathogen-free environment. The experimental protocol was approved by the ethics committee of Lanzhou University Second hospital. MKN45 cells with YWHAG knockdown and MKN45 cells were injected subcutaneously into the left side of BALB/c nude mice (five mice per group). NCI-N87 and NCI-N87 cells with YWHAG overexpression performed the same models. After one week, the tumor volumes (length×width^2/2) were measured every 3 days. On 35th day, all mice were sacrificed under deep anesthesia, and tumors were removed.

Statistical analysis

The results are analyzed using GraphPad Prism8 software. One-way analysis of variance (ANOVA) was used to analyze the differences between multiple groups, and student's *t*-test was for differences between two groups. Chi-square test was applied to analyze the correlation between YWHAG and clinicopathological characteristics. $P < 0.05$ was considered statistically significant.

Results

YWHAG is overexpression in GC tissues

To identify YWHAG expression in GC, we detected the expression of YWHAG in 107 pairs of GC and adjacent tissues by IHC. IHC results showed that YWHAG was mainly localized in the cytoplasm of human GC cells (Fig. 1A). YWHAG was significantly upregulated in GC tissues compared with normal tissues ($P < 0.0001$) (Fig. 1B). Based on the TCGA database, the level of YWHGA expression in GC was higher than that in normal samples ($P < 0.0001$) (Fig. 1C). According to the median value of YWHAG IHC score, the 107 patients with GC were divided into two groups (low or high YWHAG) to perform correlation analysis. As shown in Table 1, increased YWHAG was significantly correlated with tumor differentiation degree, whereas not significantly correlated with other clinicopathological features. These findings suggest that upregulation of YWHAG expression is very likely to be associated with malignant progression of GC.

Increased YWHAG expression promotes proliferation in GC

YWHAG expression in 6 GC cell lines was detected. The results indicated higher expression of YWHAG in MKN45 cell line and lower expression in NCI-N87 cell line compared with GES-1 (Fig. 1D). We silenced

YWHAG in MKN45 cells with lentiviral vector, and upregulated YWHAG level in NCI-N87 cells with lentiviral vector overexpressing YWHAG. Sh-YWHGA-1 and sh-YWHGA-2 significantly down-regulated the level of YWHAG in MKN45 cells (Fig. 1E). The overexpression efficiency in NCI-N87 cells was shown in Fig. 2B. We also carried out functional experiments and found that the proliferation was markedly inhibited after YWHAG knockdown, while YWHAG overexpression promoted the proliferation (Fig. 2A). In addition, EdU assay was consistent with CCK8 results, indicating that YWHAG could notably promote GC proliferation (Fig. 2B, C).

YWHAG knockdown promotes apoptosis and induces cell cycle arrest of GC cells

YWHAG had an influence on apoptosis in GC. YWHAG knockdown induced the increase of apoptosis in comparison with sh-NC group (Fig. 2D), while YWHAG overexpression inhibited apoptosis in NCI-N87 cells (Fig. 2E). To determine whether YWHAG influenced cell cycle, flow cytometry was applied. As presented in Fig. 2F, YWHAG knockdown presented strongly cell cycle arrest at G0/G1. In contrast, cells at G1 phase with YWHAG overexpressed decreased significantly.

YWHAG enhances cell migration and invasion in GC

A wound-healing assay was applied to investigate whether YWHAG affected migration. YWHAG knockdown inhibited the migration of MKN45 cells, and we observed the opposite results in YWHAG overexpression group (Fig. 3A, B). The results of the trans-well assay indicated that YWHAG enhance the invasion of GC cells (Fig. 3C, D).

YWHAG knockdown inhibits GC cells tumorigenesis in vivo

To investigate how YWHAG regulates the progression of GC in vivo, we infected MKN45 cells with sh-NC or sh-YWHAG-1 lentivirus and transferred NCI-N87 cells with YWHAG-overexpression and empty vector. Then they were used to construct a subcutaneous tumor-forming model of nude mice. Results showed that the loss of YWHAG led to a significantly lower tumor growth rate and tumor volume than the control group (Fig. 3E). In contrast, tumor growth was significantly accelerated in the up-regulated YWHAG group (Fig. 3F). In addition, we observed a decrease in Ki67 expression in the YWHAG knockdown group (Fig. 3G). In conclusion, YWHAG accelerate tumor growth of GC in vivo.

YWHAG activates PI3K/AKT pathway to facilitate cell cycle and EMT

GSEA analysis was performed to explore the functions of YWHAG in regulating GC progression, which demonstrated that high YWHAG expression was enriched in cell cycle, pyrimidine metabolism and p53 signaling pathway (Fig. 4A, B). To understand the mechanism by which YWHAG contributed to the progression of GC, cell cycle regulators were detected. Cyclin D1 and CDK2 levels were found to decrease in the YWHAG knockdown group but up-regulated in the YWHAG overexpression group (Fig. 4C, D). These

results suggested that YWHAG can effectively inhibit the expression of CDK2 and cyclin D1 to arrest cell cycle at G0/G1. Besides, EMT participates cell invasion and metastasis. Thus, we speculated that YWHAG might affect EMT in GC. As presented in Fig. 4E, YWHAG knockdown was found to enhance E-cadherin expression and decrease the expression of mesenchymal phenotypic related protein vimentin, suggesting that YWHAG knockdown can inhibit EMT. In contrast, YWHAG overexpression promoted EMT (Fig. 4F). The above findings suggested that YWHAG may regulate cell cycle and EMT process of GC cells, which is related to the characteristics of cancer metastasis. Then we applied Western blotting to evaluate whether YWHAG affected cell cycle and EMT via PI3K/AKT. The results revealed that in the YWHAG knockdown group, phosphorylation levels of PI3K and AKT significantly increased and we observed the opposite results in the YWHAG overexpression group (Fig. 4G).

Discussion

Current understanding suggests that YWHAG is involved in the progression of malignancy. Raungrut et al. showed that the malignant degree was positively correlated with YWHAG in lung cancer[11]. YWHAG is up-regulated in different cancers and mediates oncogenic transformation[6, 7, 9, 12]. We revealed the expression pattern of YWHAG and its clinical correlation in GC. YWHAG was elevated in GC and had a high risk of poor differentiation. In this study, YWHAG enhances proliferation, migration and tumorigenicity in GC while inhibiting YWHAG had the opposite effect. Further studies showed that YWHAG could also play a carcinogenic role by accelerating G0/G1 cell cycle transformation and promoting EMT.

One of the main functions of YWHA protein is to regulate cytoskeleton remodeling and migration. YWHAG could regulate the formation of actin fibers to remodel the cytoskeleton[13]. It has been reported that cytoskeleton mediated cell migration and invasion[14]. Our study demonstrated that YWHAG knockdown significantly inhibited cell invasion and migration in GC. In tumor microenvironment, hypoxia and TGF-β stimulate the expression of specific transcription factors Snai1, Slug, Twist, and ZEB1 to initiate the process of EMT[15]. Compared with benign tumors, higher grade and more aggressive tumors have more YWHAG/Snai1 complex expression[16]. Considering the critical role of YWHAG in the EMT, the expression of EMT-related proteins was detected. The results indicated that YWHAG knockdown led to the inhibition of EMT. However, whether YWHAG influences EMT progression by forming a complex with Snai1 still needs further verification. In this study, we found that YWHAG regulated the cell cycle of GC mainly by affecting G0/G1 phase. Dar et al. reported that YWHAG deficiency led to the accumulation of Set8, a substrate of the cell cycle regulator Cdt2, which delayed the progression of the G2/M phase and inhibits cell proliferation[17]. Our study did not show that YWHAG affected the progression of G2/M phase of GC.

The PI3K/ AKT signaling pathway, a frequently deregulated pathways in cancer, exerts extremely important functions in cell proliferation, angiogenesis, metastasis, autophagy and other processes[18–20]. For glioma stem cells, abnormal PI3K/ AKT signaling pathway activation can inhibit tumorigenicity[21, 22]. The PI3K/ AKT signaling pathway can medicate cisplatin resistance in GC[23, 24].

It has been reported that miR-107 enhanced the capacities of proliferation, migration and invasion by targeting FAT4 in PI3K/AKT pathway[25]. Furthermore, lncRNA AK023391 activates PI3K/AKT to promote GC cells tumorigenesis[26]. Prior studies showed that YWHA family was significant for balancing PI3K/ AKT protein network[27]. In lung cancer, YWHAG activates PI3K/ AKT pathway to functions as an oncogene[28]. In hematopoietic tumors, IL-3 activates the PI3K signaling pathway by inducing YWHAG overexpression, thereby regulating cell cycle progression, growth and apoptosis[29]. Besides, the cell cycle process could be regulated by PI3K/ AKT signaling pathway[30, 31]. Thus, the downstream cell cycle factors activated by PI3K/ AKT pathway facilitate proliferation and tumor progression. YWHAG inhibits cell apoptosis by interacting with TSC2 protein, a key mediator of the PI3K pathway[28]. Studies have revealed that angiopoietin-like 4(AngPTL4) interacts with YWHAG via the PI3K/ AKT signaling pathway and finally activates the transcription factor STAT3 to stabilize EMT-related proteins and affect EMT capacity and metastasis[16]. We used Western blotting to detect whether YWHAG knockdown inhibited PI3K/ AKT pathway activation. We found that YWHAG knockdown significantly reduced phosphorylated PI3K and AKT protein levels, which proved that YWHAG could be involved in the PI3K/AKT signaling pathway. However, whether YWHAG activates this signaling pathway through its related key downstream genes of PI3K requires to be further studied. We concluded that YWHAG participates proliferation and EMT in GC, and these effects may be due to PI3K/ AKT signaling. However, whether YWHAG has upstream or interaction will also be a research hotspot in the future, such as YWHAG and AngPTL4.

In conclusion, YWHAG is overexpressed in GC patients and may stimulate proliferation and migration via PI3K/ Akt signaling pathway. Therefore, targeting YWHAG is expected to be a crucial therapeutic option for the precise treatment in human GC.

Declarations

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Competing Interest The authors have no relevant financial or non-financial interests to disclose.

Author contribution: All the authors contributed to the study conception and design. Yanmei Gu and Daijun Wang performed the experiments, analyzed the data; Xiaomei Li, Yingying Wang and Meixia Deng assisted in the experiments; Yumin Li conceived of the study and coordinated the study. All authors gave final approval for publication.

Ethical approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Lanzhou University Second hospital (Date 2021-3-18/No 2021A-153) (Date 2021-3-17/No D2021-136).

Conflicts of interest: The authors declare that they have no conflict of interest

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Tables

Table 1. Correlation of YWHAG expression with clinicopathologic features

Characteristics	YWHAG low expression	YWHAG high expression	Statistics	*P*-value
Age (years)			0.126	0.723
≤60	26	38		
>60	16	27		
Gender			0.201	0.654
Male	28	46		
Female	14	19		
Lauren classification			2.492	0.288
Intestinal	22	24		
Diffuse	12	25		
Mixed	8	16		
Differentiation				
Poor	17	42	8.02	0.018
Moderate	23	23		
Well	2	0		
Invasion depth			2.179	0.536
T1	7	11		
T2	8	8		
T3	6	16		
T4	21	30		
Lymph node metastasis			0.81	0.847
N0	14	18		
N1	12	21		
N2	9	12		
N3	7	14		

Figures

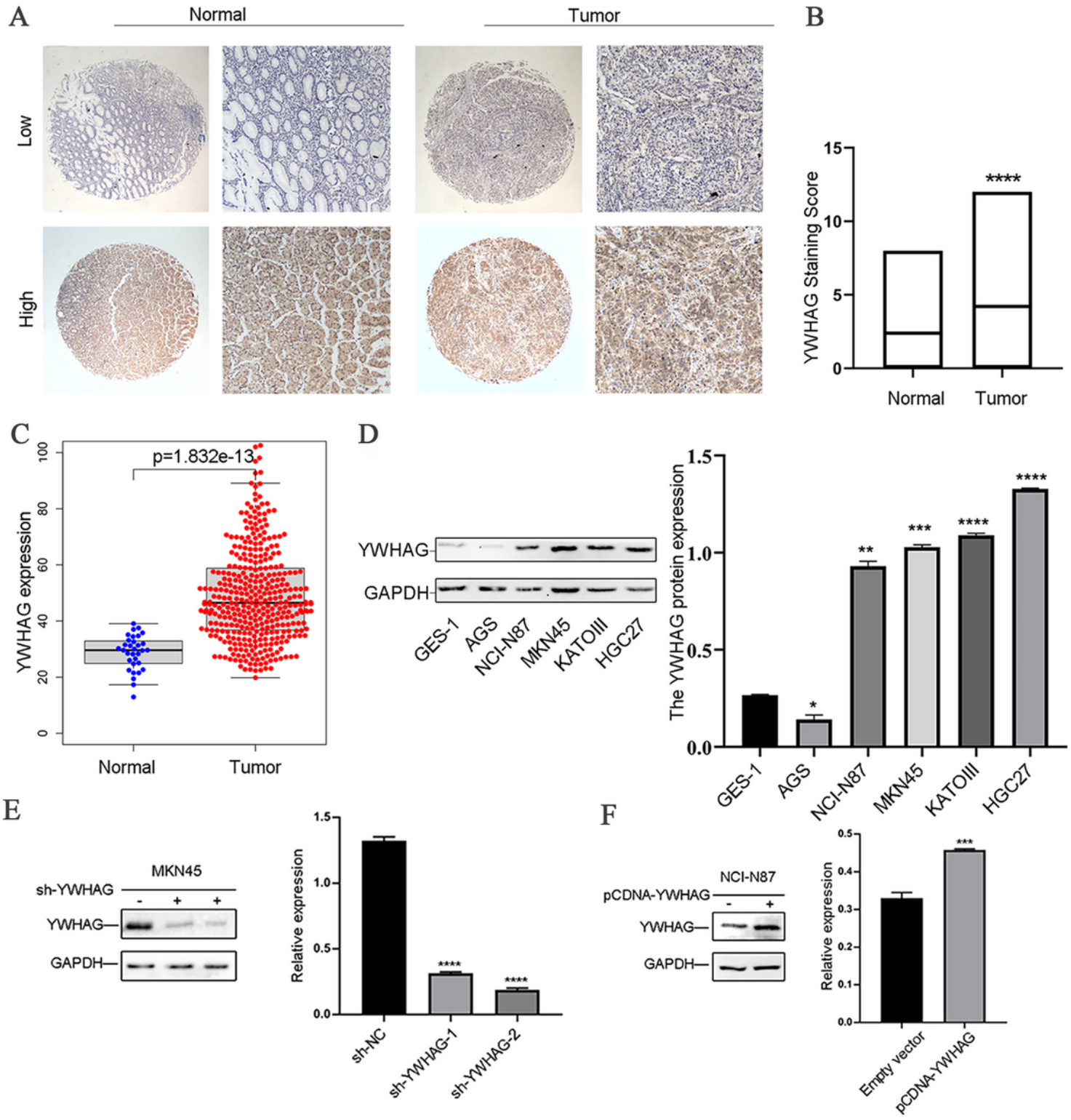

Figure 1

Expression of YWHAG. (A) Representative images for YWHAG in GC and normal tissues. (B) YWHAG upregulation in GC tissues. (C) YWHAG mRNA expression of GC in TCGA database. (D) YWHAG expression in the GC cells was determined by Western blotting. (E, F) YWHAG knockdown and overexpression efficiencies were verified by Western blotting.

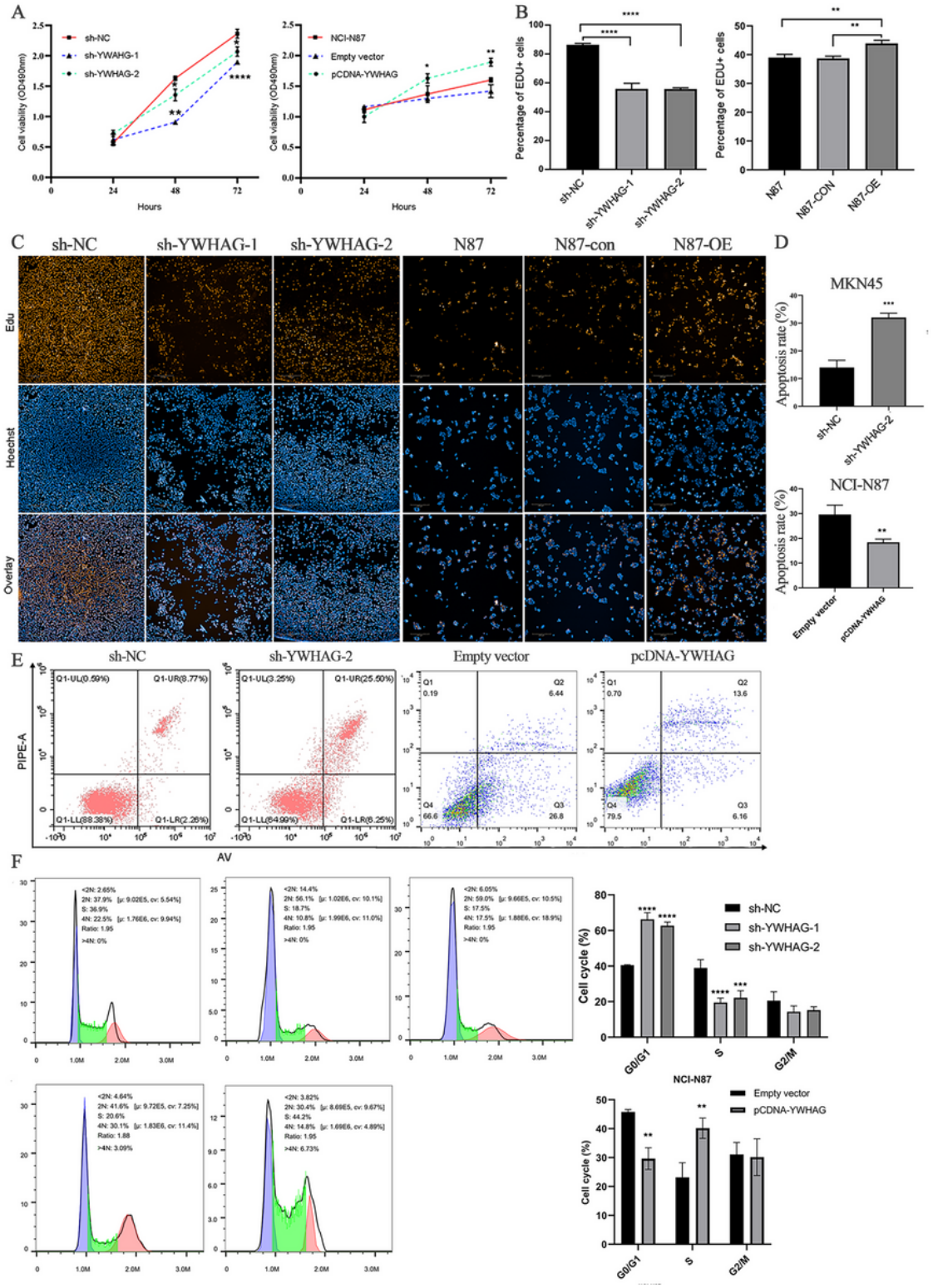

Figure 2

YWHAG promoted the proliferation of GC cells. (A) CCK8 assays revealed the proliferation of MKN45 and NCI-N87 cells. (B) Statistic for EdU assays. (C) EdU assays of YWHAG knockdown and overexpression cells. (D) Statistic for apoptosis. (E) YWHAG knockdown induced apoptosis in MKN45 cells and YWHAG overexpression reduced apoptosis. (F) Cell cycle analysis of MKN45 and NCI-N87.

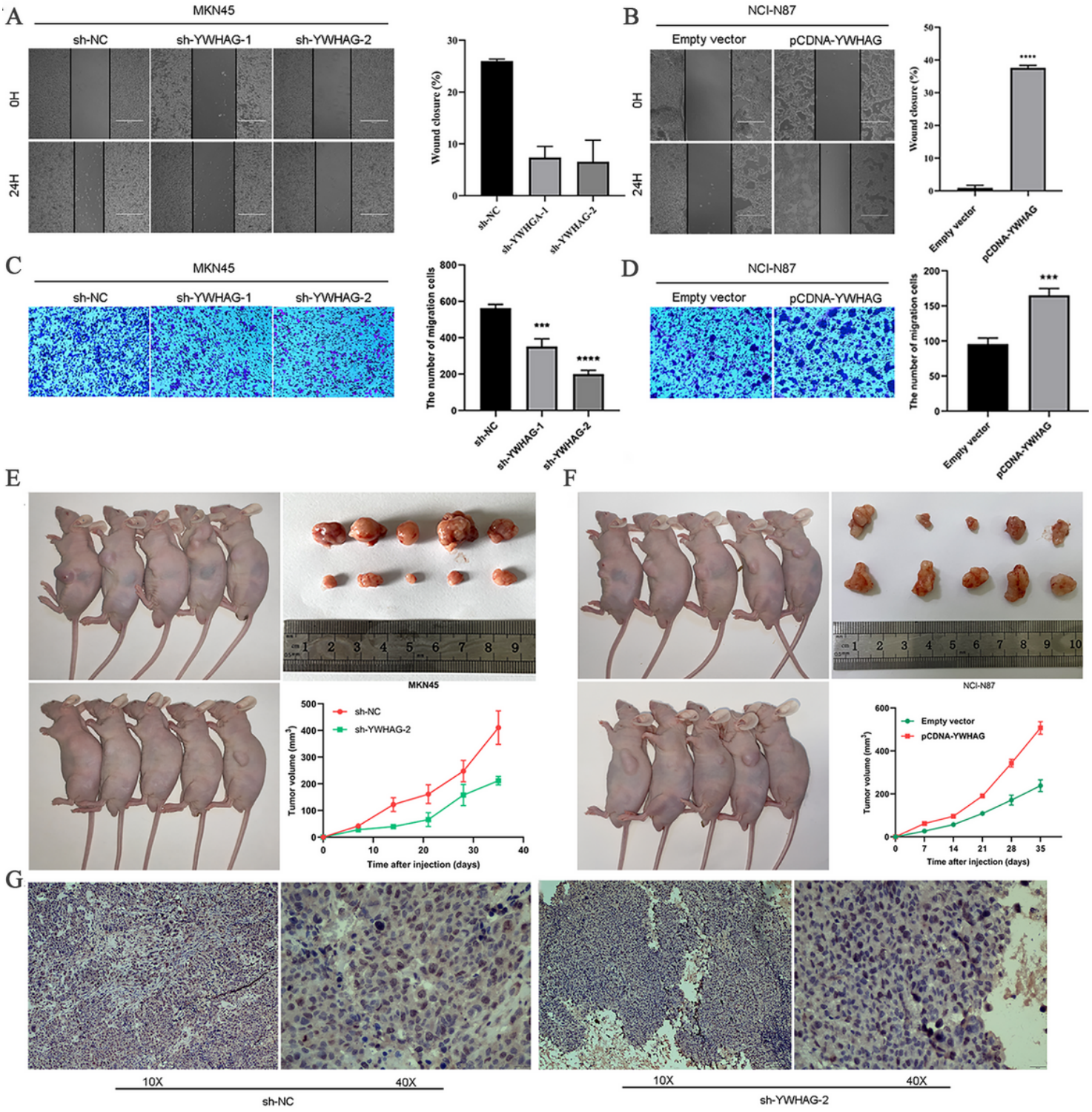

Figure 3

YWHAG promoted the invasion of GC. (A, B) Wound-healing assay in MKN45 and NCI-N87 cells. (C, D) The trans-well assay in MKN45 and NCI-N87 cells. (E) YWHAG knockdown cells formed smaller tumors compared with the sh-NC. (F) YWHAG overexpression cells formed larger tumors compared with the control group. (G) Ki67 expression in MKN45 xenograft tumors.

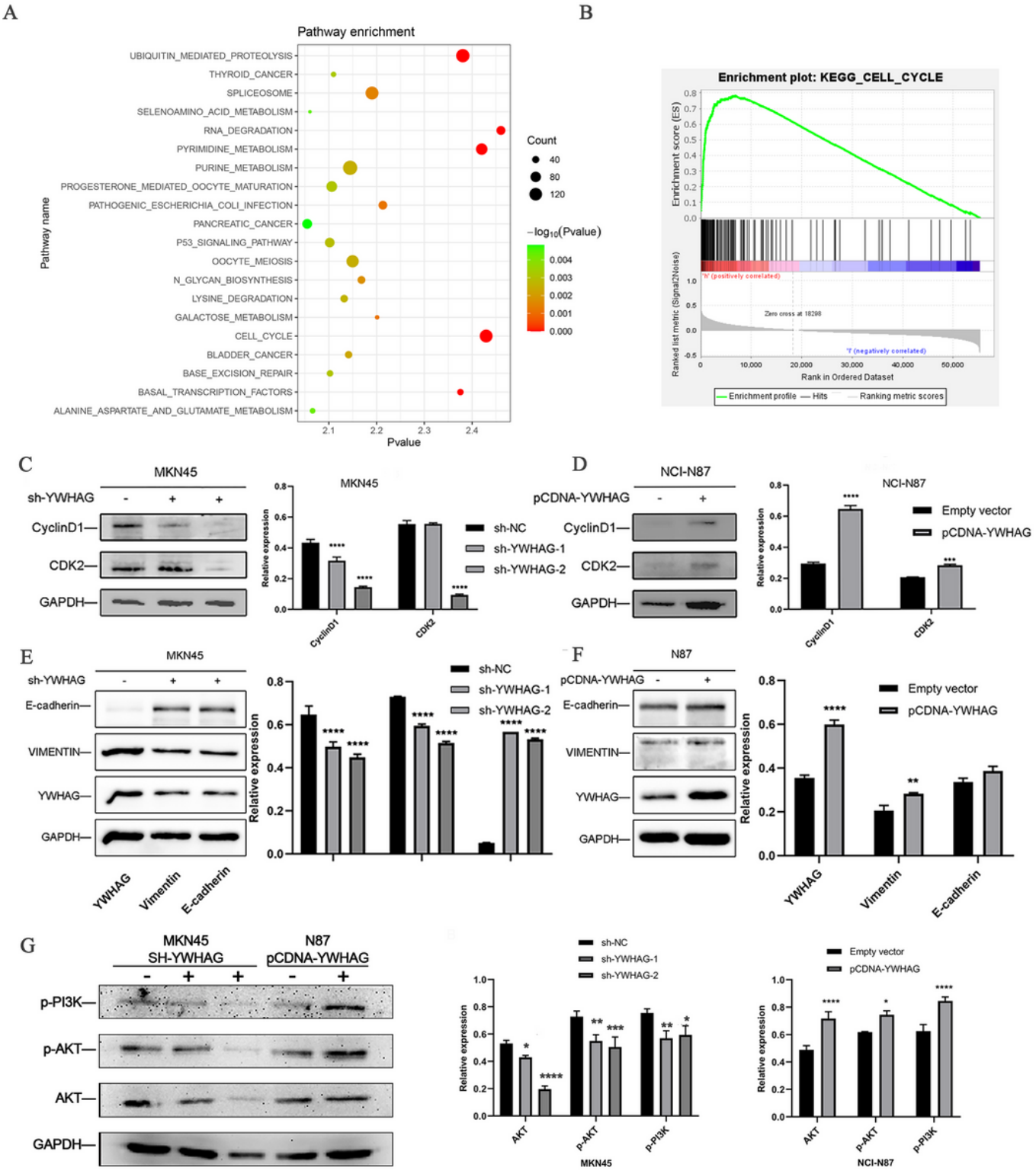

Figure 4

Mechanisms of YWHAG in GC cells. (A) GSEA of YWHAG in GC. (B) YWHAG was enriched in the cell cycle. (C, D) Expression of cell-cycle regulators in MKN45 and NCI-N87 cells. (E, F) The expression of E-cadherin and vimentin in MKN45 and NCI-N87 cells by Western blotting. (G) The expression of AKT, p-PI3K and p-AKT in MKN45 and NCI-N87 cells by Western blotting.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- animal1.jpg
- animal2.jpg
- human.jpg